Title Cryo-stress in pine seeds

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CRYO-STRESS IN PINE SEEDS

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ABSTRACT

The rapid decrease in seed viability requires new storage methods. Conventional cryopreservation /storage methods have been widely used by researchers for seed survival studies. In this study, optimum protocols were determined and investigations made on the successful cryo-storage of the three pines (*P. pinea, P. pseudostrobus* and *P. caribaea).* The results show that (i) moisture content of orthodox seeds, in pines, especially *P. pinea* seeds is not a major determinant of survival; (ii) pre-storage treatments (stepwise freezing treatments) proved to be the best optimum protocols as compared to the conventional plunge in liquid nitrogen; (iii) leakage in *P. caribaea* seeds did not indicate any stressing of those seeds whilst in *P. pinea* seeds leakage appeared to be a stress marker; and (iv) orthodox seeds or embryos can be cryopreserved at 5 – 13 % moisture contents, whilst storage between moisture contents of 15 – 26 % or more will be lethal, as shown by differential scanning calorimetry (DSC) studies on *P. pinea* embryos in this study.

1.1. CHAPTER ONE

1.1 General introduction

Pines are very important, and very often dominant, components of the vegetation over large parts of the northern hemisphere. Besides having major economic value as sources of timber, pulp, nuts, resin and other products, pines also influence ecosystems in many ways. They affect biogeochemical processes, hydrological and fire regimes, and provide food. Pines have been cultivated in many parts of the world, both within and well outside their natural range and they form the foundation of exotic forestry enterprises in many southern hemispheres. *Pinus* is without doubt the most ecologically and economically significant tree genus in the world (Bonan *et al.,* 1992). In pine (*Pinus)* species the recent progress in somatic embryogenesis, the production of genetically modified plants and the efforts towards plantation forestry have emphasized the need for germplasm conservation with functional cryopreservation protocols (Haggman *et al.,* 2000; Haggman *et al.,* 2001; Haggman *et al.,* 2006; Park, 2002).

A cryopreservation protocol had been developed for scot pine (*Pinus sylvestris*) using a classical method that involves the pre-treatment of the material and a slow cooling down to a defined prefreezing temperature, followed by a rapid immersion in liquid nitrogen (Haggman *et al.,* 1998). Using pre-treatment solutions before and during cryopreservation has improved the development of different cryo-protocols especially in pines. For example in *Pinus pinaster* embryogenic cultures were obtained with the aim of optimizing and developing a protocol for cryopreservation of *Pinus pinaster* embryogenic tissue; dimethylsulfoxide (DMSO), polyethylene glycol 4000

(PEG 4000) and sucrose solutions were applied to an embryogenic suspension culture of 250 mg/ml thus, allowing successful recovery rate of 97 % of lines stored in liquid nitrogen (Marum *et al.,* 2004). More recently six different embryogenic cell lines of *Pinus nigra* have been successfully cryopreserved in liquid nitrogen using cryoprotectants sucrose (18 %) and dimethylsulfoxide (7.5 %); after post-thaw growth, tissue proliferation was observed in five cell lines. The survival levels after storage in liquid nitrogen reached values between 62.5 % and 100 % (Salaj *et al.,* 2007).

In the cryopreservation of pines and other orthodox related genera, drying of seeds and their optimum moisture content are some of the factors to be taking into consideration. However, drying of most orthodox seeds including *Pinus* below their optimum moisture content usually has no adverse effect on seed germination and vigour. Indeed, in *Dactylorhiza fuschii* seeds, drying to low relative humidity improved cryopreservation (Wood *et al.,* 2003) while drying *Dendrobium candidum* (Wang *et al.,* 1998) and *Plantago cordata* (Pence and Clark, 2005) seeds to moisture contents less than 12 % and 5 % respectively slowed subsequent germination, but was not exacerbated by cryopreservation.

Cooling rates at which seeds of some species are cryopreserved is another factor to be taken into account in developing optimum cryo-protocols. Most oily seeds do not require slow cooling rates with the exception of *Coffea* species (Dussert *et al.,* 1997; Dussert *et al.,* 1998) and *Onopordium* species (Gonzalez-Benito and Perez-Garcia, 2001), which benefit from the use of slow cooling rates. Furthermore, lipid-rich seeds of *Pinus pinea* (Pita *et al.,* 1998), *Agathis macrophylla* (Dickie and Smith, 1995) and *Anigozanthos manglesii*

(Meritt *et al.,* 2005) showed cryopreservation related stress when cooled rapidly.

Rehydration methods can also affect survival levels, particularly of oil rich seeds (Leprince *et al.,* 1998). Recovery after cryopreservation in oily *Coffea arabica* seeds is improved when either pre-humidified for 24 hours at a relatively high temperature (37° C) prior to transfer to germination conditions (Dussert *et al.,* 2003) or osmo-conditioned for six weeks with a -1.25 MPa solution of polyethylene glycol (PEG) (Dussert *et al.,* 2000).

Following conventional seed banking studies already done by researchers over the years, there is the need for conservation of seeds threatened with extinction (SCBD, 2002) because all seeds whether stored or not will eventually die; but at cryopreservation temperatures seed deterioration is virtually stopped (Walters *et al.,* 2004) allowing long-term preservation of these genetic resources.

The need to design new optimum protocols have been emphasized over the years to preserve and conserve these genetic resources.

There is evidence that seeds of some species, especially *Pinus pinea* (Pita *et al.,* 1998), have shown reduced germination and vigour of about 24 % after cryopreservation treatment. Such responses could be due to cooling and warming too quickly; associated with specific low temperatures; a result of ice caused by crystallization; membrane or electrolyte leakage and a moisture content above critical water content for cryopreservation. The objective of this study is to understand the mechanism of cold stress in pine seeds and to develop an optimum protocol for effective cryo-storage of the three species (*Pinus pinea, Pinus pseudostrobus and Pinus caribaea*).

1.2. Species introduction

Three species of *Pinus* were experimented upon to determine an optimum cryoprotocol to improve the conservation of oily seeds in respect to pines. These species of pine were namely *Pinus pinea, Pinus pseudostrobus* and *Pinus caribaea*.

These species vary in, biogeographical region and habitat.

Pinus pinea (Mediterranean stone, Italian stone and umbrella pine) is found on the Mediterranean coast and belongs to the biogeographical region called the Mediterranean Basin; it has a cone length of 10 - 15cm (Kindel, 1995). *Pinus pseudostrobus* (Mexican false white, false Weymouth pine and dwarf stone pine) is normally seen in the montane habitat and can be found in the south Mexican and Guatemala forests and its cone length is about 8-15 cm (Kindel, 1995). *Pinus caribaea* (Caribbean pine) is from the Caribbean area, as its name implies, and also Central America. Its most suitable habitat is in the tropics. Its cone length is 5 - 12 cm (Kindel, 1995). Table 1.1 shows common names, biogeographical region, habitat and cone length of 13 species of *Pinus* including *P. pinea, P. pseudostrobus* and *P. caribaea.*

Table 1.1.

Pinus taxon	Common name(s)	Cone length (cm)	Biogeographical region(s)	Habitat(s)
* *P. caribaea*	Caribbean pine	5 - 12	Caribbean area and Central American	tropics
P. cembra	Swiss stone or Arolla pine	4 - 10	Europe	subalpine
P. contorta	Lodgepole pine	2 - 6	Western America	temperate
P. echinata	Shortleaf pine	4 - 7	South East USA	temperate
P. edulis	Colorado pinyon	3 - 6	South West USA	arid
P. elliotii	Slash pine	8 - 18	South East USA	temperate
P. engelmannii	Apache pine	10 - 15	West Mexico, Arizona, New Mexico	temperate/montane
P. nigra	European Black, Austrian pine	3 - 10	Europe and Mediterranean Basin	temperate
P. pinaster	Maritime pine	10 - 22	West Mediterranean Basin	Mediterranean coastal
* *P. pinea*	Mediterranean stone, Italian stone, Umbrella pine	10 - 15	Mediterranean Basin	Mediterranean coastal
P. pseudostrobus	Mexican false white, false Weymouth pine, dwarf stone pine	8 - 15	South Mexico, Guatemala	tropical montane
P. radiata	Monterey pine	7 - 15	California, Baja California	Mediterranean coastal
P.sylvestris	Scots pine	3 - 6	Europe and Central Asia	Boreal forests, temperate, subalpine

N.B. Asterixed species were the ones worked upon for this project.

Source: Kindel, 1995.

1.3. Storage of seeds

Storage of seeds is arguably the most effective and efficient method for the *ex situ* preservation of plant genetic resources. Low storage costs, combined with ease of seed distribution and regeneration of whole plants from genetically diverse material, offer distinct advantages for the storage for conservation of seed compared with other types of plant tissues such as meristems and pollen (Linington and Pritchard, 2001).

Conventional seed gene banks maintain seeds at about 5 % (w/w) moisture content (fresh weight basis) and -18 °C. Under such storage conditions it is predicted that high levels of viability will be retained over many decades to centuries (Dickie *et al.,* 1990).

1.3.1. Seed characteristics in relation to moisture content

In terms of the water relations of survival, orthodox seeds tolerate the removal of both free water and most bound water (Vertucci and Farrant, 1995). As the latter is reduced, longevity is increased, at least down to moisture contents in equilibrium with around 15-20 percent relative humidity (Robert and Ellis, 1989). Other seeds clearly lose viability as bound water (less than 85 percent relative humidity) is being removed, such as some *Coffea* (Rubiaceae; Dussert *et al.,* 1999), and they tend to be short-lived in the dry state (Bonner, 1990). In contrast, recalcitrant seeds tolerate the loss of free water to around 20 percent moisture content (about 85-90 percent relative humidity), but

usually only after rapid desiccation(Pence, 1995; Vertucci and Farrant, 1995).

Detailed studies since the 1990s have revealed that the level of desiccation tolerance within a species is a consequence of a number of factors that effect a specific seed-lot, especially in relation to the developmental age of seeds and the method of handling (Black and Pritchard, 2002). It is possible that seedlots of the same species harvested, for example, in different years could respond to desiccation (and other storage treatments) in a subtly different way (Pammenter and Berjak ,1999).

In addition, the apparent level of desiccation tolerance appears to vary within a seedlot. The response of an individual seed to desiccation is all or nothing in the sense that it either does or does not subsequently germinate (i.e it passes or fails the viability test) take for instance seeds of *Aesculus hippocastanum* (Daws *et al.,* 2004) and *Acer pseudoplatanus* (Daws *et al.,* 2006) did germinate well in Southern Europe than in Northern Europe because Southern Europe is warmer than Northern Europe, hence, seeds sown in Southern Europe exhibits less dormancy and more desiccation tolerance.

Air temperature during development of seeds can affect seed dormancy; dormancy levels are typically inversely related to the heat sum (° C per day) accumulated during development, for both herbs (Fenner, 1991) and woody perennials, such as *Aesculus hippocastanum* (Pritchard *et al.,* 1999). In a number of species, seed or fruit size is positively related to increasing air

temperatures during developments (Stanley *et al.,* 2000). Furthermore, seeds of late-maturing annuals may be shed at an earlier developmental point (i.e. with smaller, less developed embryos), as a consequence of a reduced developmental heat sum (Wagner and Mitterhofer, 1998).

However, the measure of desiccation tolerance is applied at the population level. Most commonly this is assessed via co-plots of germination (on a probit or probability scale) against moisture content.

Comparison of such co-plots for recalcitrant seeds of three temperate species, using whole seed, and embryonic axis, moisture content (MC) values down to about 20 percent, reveals that dependency of survival on moisture contents is approximately 0.2 probits MC^{-1} (Pritchard and Prendergast, 1986; Pritchard 1991; Tompsett and Pritchard, 1998). Interpolation of data for six Coffea species with desiccation sensitivities down to around 10 percent moisture content (Dussert *et al.*, 1999) yield a similar value; for any 10 percent fall in moisture content, 2 probits of viability would be lost. This is equivalent to the viability falling from 84 to 16 % a range of survival often covered by desiccation sensitivity experiments.

Such variability in response to drying within the population could result from two factors: (1) seed to seed variation in desiccation tolerance per se (e.g., as a feature of differential development age) and (2) a normal distribution in seed moisture contents within the population at any sample time during drying masking a single or narrow range of critical moisture contents . Unravelling

the contribution of each factor remains a challenge (Black and Pritchard, 2002).

However, seeds can be broadly divided into three major groups, based on their sensitivity of desiccation and to low temperatures, as follows:

1.3.2. Orthodox seeds

This group includes all seeds which desiccate naturally on their mother plants. The seed can be dried to lower moisture contents (less than 10 %) (Stanwood, 1985) without any deleterious effects. In fact, the lower the seed moisture content and storage temperature the longer they survive.

1.3.3. Recalcitrant seeds

This group of seeds easily die if they are dried below certain moisture limits (12 - 30 %) (Hong and Ellis, 1996). The tropical recalcitrant seeds are also killed when exposed to low temperatures (less than 16 °C). Even in optimal moist conditions survival of seeds in this group is limited from a few weeks to a few months. A critical aspect feature for success is storing the seeds at close to the minimum temperature for germination, which varies between recalcitrant species. For example the best storage recorded performance (in percentage viability/days/° C) for both *Aesculus hippocastanum* (Baskin and Baskin, 1998) and *Quercus robur* (Buitink *et al.,* 1998; Buitink, 2000) are 40 %; 150 days; 16 ° C and 52 %; 280 days; 2° C respectively.

If they are not collected at maturity and sown immediately, they will die. The period between collection and sowing should be short. These seeds therefore are difficult to store and do not conform to the rules applicable to orthodox seeds.

1.3.4. Intermediate seeds

This is a category that has been recently defined (Ellis *et al.,* 1990). The seeds in this category have storage characteristic intermediate between orthodox and recalcitrant seeds. Intermediate seeds can be dried to seed moisture levels similar to that of orthodox seeds, although there is some viability loss below about 10 % moisture content (MC). However, the dry seeds are injured when exposed to low temperatures and viability drops rapidly while in storage. Therefore, these seeds can be stored under conditions used for orthodox seeds but possibly for only a short period of time.

1.3.5. Major difference(s) between conventional seed banking and cryopreservation

In conventional seed banking conditions viable cells, tissues and organs are held at temperatures ranging from -18 °C to -30 °C, whereas, cryopreservation is the preservation of viable cells, tissues and organs at temperatures ranging from -70 °C to -196 °C.

1.3.6. Problems associated with conventional/traditional seed banking

Generally, seed banking (usually -20 °C) is considered to be the most efficient and economic method for the conservation of genetic resources (majority of species) (Gomez-Campo, 1985; Hawkes, 1990). However for pines, traditional/conventional banking has only proven to be efficient for short term and medium term conservation. *P. elliotii, P. patula, P. radiata* and *P. taeda* after 6 years of storage at -16°C did not show a loss of viability (Donald and Jacobs, 1990). Longer periods in traditional/conventional seed banking seems to affect seed vigour and viability, as Donald and Jacobs (1990) reported that storage periods over 15 years in *P. elliotii, P. patula, P. radiata* and *P. taeda* resulted in an increase in abnormal germination (i.e. seed vigour and viability).

However, statements above shows that conventional/traditional seed banking can only be efficient for a short period of time. When this period does not exceed the natural interval between germination and seed production for the next generation, other alternatives should be considered (Bonner, 1990). For pines this means successful storage of more than 50 years depending on species.

Cryopreservation may be an alternative (Ahuja, 1986; Jorgensen, 1990; Rao and Riley, 1994; Pita *et al.,* 1997).

1.4 Cryopreservation

Cryopreservation is the preservation of viable cells, tissues and organs in extreme temperatures of -70°C to -196°C. This storage procedure can be successfully applied to a wide range of organisms and biological tissues (Benson and Lynch, 1998) and its increasingly used to conserve crop plant germplasm (Reed *et al.,* 2001). Cryopreservation provides a long term storage method for the conservation of plant genetic resources which cannot be maintained using the conventional seed banking method. However, Barnet and Vozzo (1985) noted that germination levels fell in *P. echinata* and *P. elliotii* by 25 % and 66 % respectively when 7 % and 6.28 % moisture content seeds were stored at -16 °C; there was also an observable loss of vigour and increase in chromosomal aberrations.

The above clearly denotes that seed viability and seedling vigour are only maintained for short periods in comparisons to the life span-life cycle of pines. Thus, the use of conventional seed banking storage is clearly limited and is not recommended because the potential storage period is shorter than the natural interval between germination and seed production for the next generation (Bonner, 1990). Cryopreservation has been advocated for tree seeds (Pita *et al.,* 1998). However, existing large scale cryopreservation facilities are not focussing their efforts on tree germplasm. The last decade has resulted in many outstanding developments in plant cryoprotection research most significantly, vitrification based protocols and simplified procedures have been developed by a number of researchers (Engelmann, 1994) making cryopreservation effective for a broad range of species.

the basis that biochemical processes are so reduced in cryopreserved material that biological deterioration is virtually stopped (Stanwood and Bass, 1981; Stanwood, 1985). However, evidence of improved physiological preservation of seeds when cryopreserved compared to when stored at conventional gene bank temperatures is limited. For example the loss in seed viability of an orchid species is lower at -196 °C compared with that at -20 °C (Pritchard and Seaton, 1993). Nonetheless, it is possible to estimate the theoretical benefits of adopting cryopreservation for seed storage by extrapolation from published rates of viability loss at higher temperatures (Dickie *et al.,* 1990). Based on the combined analysis of longevity data for the seeds of eight species stored at 90°C to -13°C (Dickie *et al.*, 1990), it is projected that the logarithm of seed longevity could be 2.25 units greater if liquid nitrogen rather than conventional freezer storage of about -18 °C to -20 °C is used. This means that the standard deviation of the lifespan of individual seeds in a population stored under the same moisture conditions could be about 175 times greater. In other words, the time for viability to fall by one on the probit scale (e.g from 97.7 - 84.1 %) could be 175 times longer. Thus, the relative benefits to seed longevity of adopting cryopreservation as the storage system may be considerable. It is important to note, however, that such theoretical considerations are made on two tentative assumptions. First, it is assumed that an exponential function can be used to describe the effects of temperature on the rate of seed viability loss. The alternative temperature model fitted to the data (Dickie *et al.,* 1990) includes a quadratic function that the logarithm of longevity of lettuce seeds , for example , stored at 5 % moisture content (fw) would be the same (i.e., 0 - 2 days) at -150 °C as at 80

°C. Moreover, longevity at -196°C is theoretically shorter than this , although there is no evidence for this in practice, even for relatively moist but not wet seeds (Iriondo *et al.,* 1992) .Second, it is supposed that the effects of temperature and moisture content on seed viability loss are inter-dependent (Dickie *et al .,* 1990) ,although, evidence for the existence of such a relationship down to cryopreservation temperatures is not yet available. More recently Walters *et al.,* (2004) have shown in cryopreservation the increased longevity in lettuce seeds when compared to same seeds stored at -18 °C; suggesting that cryopreserved seeds may have half times of 500-3400 years.

Accepting that there are likely to be some benefits to seed longevity through the use of cryopreservation, it is pertinent to consider which types of species are most suited to preservation in this way. Ideal candidates might be species with inherently short lived seeds and endangered species with critically small population sizes; both types of material would benefit from unlimited storage potential that liquid nitrogen storage appears to offer. However, it is not possible at this stage to recommend the use of cryopreservation over conventional seed bank storage for the conservation of bulk seed lots in a majority of desiccation tolerant or "orthodox" seeds. The practical benefits of the latter method currently appear to outweigh the potential longevity gains associated with the former.

Early indications that seeds could tolerate cryopreservation treatment are referred to more fully in a comprehensive review of the subject (Stanwood, 1985). Interestingly, work on seed cryopreservation goes back more than 100 years ago showed that seeds of 12 species from 8 families

were shown to survive 110 hours at -183 °C to -192 °C (Brown and Escombe,1897-1898), In that study, air-dried seeds were used at a natural moisture level of 10 - 12%. In the intervening period it has been demonstrated that dry seeds of about 300 species tolerate exposure to liquid nitrogen (Stanwood and Bass, 1981; Stanwood, 1985; Sakai and Noshiro, 1975; Touchell and Dixon, 1993) including representatives of some important plant families such as palms (Al-madeni and Tisserat, 1986) and orchids (Pritchard and Seaton, 1993; Pritchard, 1984). Moreover, it is expected that a majority of orthodox seeds of other species will respond favourably to cryopreservative treatment.

There are plant species for which storage, under conventional or cryo-storage conditions, is not feasible, for example species, that do not readily produce viable seed. For these, meristem cryo-storage is an alternative strategy for *ex situ* preservation. Also, many woody perennials produce large fleshly seeds that are intolerant of drying to low seed moisture content (<20 %). Techniques are being developed for the cryo–storage of the embryo, including the excised embryonic axis (Pritchard and Pendegrast, 1986 ; Pence, 1992). An alternative approach to the problem of recalcitrant seed storage has been to simulate their response to cryoprotection and freezing using orthodox seeds techniques (Grout, 1979; De Bouchard and Cambecedes, 1988). However, the validity of this approach is uncertain; a recalcitrant seed is unlikely to have undergone the same physiological and biochemical modification associated with early stages of orthodox seed maturation drying. Nonetheless, this approach to the cryo- storage of the

orthodox seed was addressed here. In addition, this chapter summarizes the main factors that need to be controlled to achieve the successful cryo-storage of dry seeds: seed moisture content and the cooling/warming regimes used . Finally attention was given to the methods for assessment of seed quality following cryo-storage.

1.4.1 The need for cryopreservation

The general interest in cryopreservation studies has increased significantly recently (Pritchard, 2002). Whilst it was suggested previously that candidate species for dry cryopreservation were those with inherently short-lived seeds and endangered species with critically small population sizes (Pritchard, 1995), a review of seed cryopreservation studies from 1995 to 2005 reveals considerable interest in socio-economically-important species particularly woody and horticultural species.

Seed deterioration is virtually stopped at cryopreservation temperatures (Stanwood, 1985; Pritchard, 1995; Walters *et al.,* 2004). An earlier forecast suggested that seed longevity at cryogenic temperatures could be about 175 times longer than at conventional seed bank temperature (Pritchard, 1995). Comparable estimates for lettuce seed longevity at -18 °C, based on the seed viability calculation, are up to 46 - 70 years, i.e. up to 74 times less than cryogenic temperatures (Walters *et al.,* 2004).

Moisture contents at which seeds have been cryopreserved have varied from about 7 to 14 %, depending on species and the seed oil content. In many species the optimum is around 10 % moisture, e.g. in *Citrus* (Lambardi *et al.*, 2004), *Azadirachta indica* (Berjak and Dumet, 1996), *Piper* (Decruse and Seeni, 2003) and *Warburghia salutaris* (Kioko *et al.*, 2000). Moisture content close to this optimum or just above are likely to be close to the high moisture freezing limit (HMFL) (Stanwood, 1985) increasing the risk of small quantities of ice forming during cooling/warming. This risk seems to be higher in lipid rich seeds; lipid thermal transitions possibly enable nucleation of small ice crystals forming into larger pernicious ones (Dussert *et al.*, 2001). It is hypothesized that the interaction between lipid and water during freezing is responsible for the formation of ice crystals that were large enough to cause lethal damage (Vertucci, 1989); and perhaps the reason why *P. pinea* showed stress in germination (Pita *et al.*, 1998).

1.4.2. Cryopreservation of orthodox seeds with reference to pines

The results from studies carried out by Pita *et al.* (1998) on seven native Spanish species of pines are shown on Table 1.3

Specie(s)	Control (germination %)	Storage in Liquid nitrogen for 4 days (germination %)	Moisture content (%)
P. canariensis	86	91	9.57
P. halepensis	90	84	7.63
P. pinaster	94	88	9.15
* *P. pinea*	85	61	9.82
P. nigra	97	96	7.28
P. uncinata	70	71	7.26
P. sylvestris	100	97	7.71

*Only in *P. pinea* was there a notable difference when sown before and after storage in liquid nitrogen.

1.4.3. Seed deterioration and storage environment

Orthodox seeds in air-dry storage deteriorate. Eventually this deterioration results in loss of viability of individual seeds. Within an accession individual seeds die at different times. Consequently the viability of an accession is assessed as the proportion (expressed as a percentage) of viable seeds. The proportion of live seeds within an accession is also an indicator of the accumulation of deterioration within seeds which remain alive. For example as percentage viability declines the time taken by surviving seeds to germinate

increases quite markedly (Ellis and Roberts, 1980) and the surviving seeds are also more likely to fail to germinate in sub-optimal environments (Ellis and Roberts, 1981). There is also, however, a more subtle aspect to this deterioration within the surviving seed that must be noted for genetic resources conservation; loss of viability is correlated with an accumulation of chromosome damage in surviving seeds (Murata *et al.*, 1981) and with heritable damage in succeeding generations (Abdalla and Roberts, 1969).

Although seed deterioration in air-dry storage can thus be considered as a form of ageing, it does not only occur as a function of time. The rate of deterioration is a positive function of both seed moisture content and temperature. Thus deterioration occurs relatively slowly at low moisture and temperature, which is why the preferred conditions for long term storage are considerably cooler and drier than those of commercial practice – that is cryopreservation of these seeds are preferred to conventional seed banking.

1.4.4. Seed deterioration in relation to membrane damage in seeds

According to Wilson Junior and McDonald Junior (1986) the deterioration process would have as a consequence the destruction of the membrane systems at a cellular level with lipid oxidation being the direct cause of the seed deterioration during storage. Furthermore, within the lipid fraction, the polyunsaturated fatty acids such as linoleic and linolenic are the most important and susceptible to to oxidative degradation.

Oxidation of the lipid present in the cell membrane and the concomitant increase in the concentration of free fatty acids has been proposed as the main cause of seed deterioration. The oxidative attack generally begins on the polyunsaturated fatty acids (linoleic and linolenic) found in the plasmatic membrane. Thus, the first step in seed deterioration would be the loss of the membrane integrity, leading to an increase in its permeability and causing leakage of cellular solution during soaking and storage (Wilson and McDonald, 1986). In soybeans there were high levels of malondialdehyde, a product of the peroxidation of unsaturated fatty acids and also levels of linoleic (18:2) and linolenic (18:3) acids in a phospholipid decrease during aging and deterioration of those seeds. Walters *et al.,* (2002) discovered that *Cuphea carthagenesis,* an orthodox seed, was sensitive to -18 °C storage and showed stress in germination after harvested. A comparison of lipid composition among *Cuphea* accesions from 15 species suggests that those susceptible to damage on exposure to -18°C storage are those with shorter-chain unsaturated fatty acids while those that are not susceptible to -18°C are those with longer-chain unsaturated fatty acids.

1.4.5. Seed deterioration in relation to electrolyte leakage and ageing

The loss of seed viability can be associated sometimes with large increase in the release of solutes and electrolytes within the system there by bringing about the excessive production of fatty acids like malondialdehyde into the embryonic axis and cotyledons of seeds, suggesting a loss of membrane integrity probably due to lipid peroxidation and change in the composition of those fatty acids.

The increase in the production electrolytes and solutes like potassium (K^+) might be due to increase thawing or intense heat applied before imbibition prior to sowing takes place. For example sunflower seeds (*Helianthus annuus*) heated at 45 °C before imbibed in water for 72 hours were all found to be dead (Corbineau *et al.,* 2002).

In scots pine (*P.sylvestris)* seeds (Tammela *et al.,* 2005) ageing is factor that leads to declined vigour and poor germination. This is because ageing induces changes in fatty acids composition and bring about biochemical changes that are associated with increased electrolyte leakage.

1.4.6. Applications of differential scanning calorimetry

Differential scanning calorimetry (DSC) is increasingly used to measure freezing events in plants, including seed tissues (Pritchard and Manger, 1998). The differential scanning calorimeter is a sensitive instrument that can be used to monitor thermal transitions in samples of tissue, embryo and endosperms. For example thermal transitions are associated with the formation of intracellular ice upon cooling, and recrystallisation upon rewarming, which are the main causes of lethal injury leading to mortality during storage in liquid nitrogen (Martinez and Revilla, 1998).

The main cryobiological application of differential scanning calorimetry (DSC) is to analyse the physical state of water during cooling and heating cycles to assist in the development of vitrification based methods (Benson *et al.,* 1996b).

Data are collected as a function of time and temperature. Pretreated samples of known weight are sealed into an aluminium pan and placed inside the DSC chamber alongside an unloaded reference pan; both pans are simultaneously cooled or warmed in at a programmable rate to a prescribed final temperature. Differences in heat flow between two pans is measured during cooling and heating and the software plots this function against temperature or time to produce differential heat thermograms.

Thermal events, such as the latent heat of fusion alter the amount of heat required to maintain both pans at the same temperature, causing a change in the heat flow and this differential deflection in the thermogram is directly attributed to thermal transitions in the sample. Transitions between liquid, ice and amorphous glassy states can be detected from heat flow data shown as an exothermic peak during cooling and an endothermic peak during rewarming of samples.

The point of change from liquid to glass is the glass transition (Tg) temperature, which is detected as subtle deflection in heat capacity. The glass is a highly viscous metastable amorphous state and its properties depend on the final temperature of transition and sample moisture content (Mazur, 2004).

There are two components during cooling that do not form ice in the supercooled state: water associated with the glass transition and water that remains unfrozen i.e. is bound or osmotically inactive (Wolfe *et al.*, 2002). Therefore, many researchers have used DSC to examine the physical

changes occurring during cryogenic treatments (Hor *et al.,* 2005; Dumet *et al.,* 2000b; Kim *et al.,* 2005; Dussert *et al.,* 2001; Martinez, 1998; Vertucci *et al.,* 1991). DSC can also be used to detect thermal transitions in lipids.

1.5. Aims and objective of project

The major aim of this study was to determine the sensitivity of oil rich pine seeds to cryopreservation; optimise protocols with a view to achieving successful cryopreservation of *Pinus pinea, Pinus* pseudostrobus and *Pinus caribaea.* Hence the approaches and objectives of the studies were to :

(I) Determine the effects of six different storage temperatures on the three different species (*Pinus pinea, Pinus pseudostrobus* and *Pinus caribaea*) since especially *Pinus pinea* has been shown to have reduced in vigour and germination after cryopreservation (Pita *et al,* 1998)

(II) Determine leakage in pines (*P. pinea* and *P. caribaea*) using CM100 conductivity meter, to find out whether leakage in pines is a reliable marker of stress after cryopreservation.

(III) Determine ice crystallisation and oil transitions in *P. pinea* using differential scanning calorimetry (DSC).

(IV) Identify optimum cryopreservation protocol in all three pines (*P. pinea, P. pseudostrobus* and *P. caribaea*).

CHAPTER TWO

2.0. MATERIALS AND METHODS

Introduction

To establish the effects of cryopreservation of pine seeds; four basic approaches were undertaken. These were:

(i) Seed moisture content determination tests determining moisture content of seeds before experimentation on cryopreservation.

(ii) The effects of six different cryopreservation protocols on the three different species especially *Pinus pinea*.

(iii) Differential scanning calorimetry to determine solidification and melting transitions at different cooling and warming rates, with graphical thermograms.

(iv) Conductivity tests, to establish whether cryopreservation affected the level of leakage from seeds, to determine if this form of test could be used as an indicator for the reduced viability in the seeds.

All experimental work took place in the Seed Conservation Department of the Royal Botanic Gardens, Kew, Wakehurst Place.

2.1. Seed material

Seeds of *Pinus pinea, Pinus pseudostrobus* and *Pinus caribaea* were found and collected at Italy; Mexico and Jamaica respectively. They were brought into seed bank in the following year(s): *P. pinea* – 1995; *P. pseudostrobus* – 1983 and *P.caribaea* – 1983. Both *P. pseudostrobus* and *P. caribaea* seed-lots were stored in conventional storage freezers of 20° C for three years before transferred to dry room. Seed-lots of all three species were stored in a dry room at 15 % relative humidity in sealed plastic bags. Seeds of the three species were weighed and numbers of seeds were calculated. Seeds were taken from the dry room to the laboratory before each test was performed. To avoid seeds absorbing moisture, Glovco hand gloves were worn and seeds sealed in plastic bags until use.

2.1.1. Seed weight

The total weights of the *Pinus pinea, Pinus pseudostrobus* and *Pinus caribaea* seed-lots were 946.4 g, 23.8 g and 688.2 g respectively, equating to 1245, 793 and 17205 seeds respectively.

Room temperatures in the laboratory, cryopreservation rooms and dry room were approximately 20 °C-23 °C, 20 °C and 15 °C respectively.

2.2. Initial seed tests

2.2.2. Initial moisture content tests

Seed moisture content was determined for each seed-lot by carefully selecting ten seeds of each that were free of punctures and cracks.
Fresh weights of each seed were taken on a balance after which all samples were placed in oven of 103 °C for 17 hours (ISTA, 1999); each seed being

labeled from 1 - 10. To get the dry weight, seeds were removed from the oven and placed over silica gel to cool for 30 minutes and then reweighed.

Moisture content (MC) was calculated as:

$$MC = (W2 - W3)/ (W2 - W1) * 100\%$$

Where W2 = fresh weight of sample + dish weight

W3 = dry weight of sample + dish weight

W1 = weight of dish

All percentage (%) moisture contents are presented on fresh weight basis for whole seeds throughout the thesis.

2.2.3. Non-destructive relative humidity tests

The purpose of this test is to determine moisture contents at which different percentage relative humidities are achieved, deriving isotherms for different seeds especially pines and to establish the fact that seeds with higher oil contents will have lower moisture content at same relative humidity.

Relative humidity (RH) of the three seed samples was measured using a Rotronic hygrometer: Rollog Agent-HT1 unit with HW3 software (Rotronic Limited, Crawley, UK).

Seeds were placed on Rotronic probe containers 1 and 2 filling both probes to the brim and closing both containers: with *P. pinea* having five seeds per probe and both *P. pseudostrobus* and *P. caribaea* ten seeds per probe.

Samples were measured in triplicates and seeds were allowed to equilibrate for at least 30 minutes and at most 1 hour.

2.2.4. Germination tests

Four replicates of twenty seeds were placed on 1% agar medium in sandwich boxes (17 cm×11 cm×10 cm) . Seeds were incubated at 20 °C on a day/night cycle of 8 hour/16 hours with an irradiance of 15 μ mol m^{-2} s^{-1} provided by cool white fluorescent tubes (OSRAM L 58W/ 20).

Seeds were scored each for 28 days and percentage germination was calculated for each species based on radicle emergence by 1 mm. All seed emerging with radicles developed into seedlings.

Cryopreservation comparisons were made before and after storage on all three species. Cooling and stepwise protocols used for the treatments were as follows:

(i) T1 – Rapid cooling.

(ii) T2 – One step cooling.

(iii) T3 – One step cooling.

(iv) T4 – Three step cooling before direct plunge into liquid nitrogen.

(v) T5 – Two step cooling before direct plunge into liquid nitrogen.

(vi) T6 – One step cooling before direct plunge into liquid nitrogen.

It is important to note that for all protocols shown above that all samples of seeds in their aluminium sealed bags were allowed to thaw on laboratory bench for 24 hours. Thawing technique was based on the work of Pita *et al.,* 1998, where seeds were allowed to thaw for 24 hours on laboratory bench.

This protocol/experiment was repeated for the six storage treatments for the species used in this project. The treatments are as shown in Table 2.1 below:

TREATMENT	TREATMENT(S) DESCRIPTION
T1	Direct plunge in liquid nitrogen (-196 °C) for 4 days
T2	Storage in -50 °C freezer for 4 days
T3	Storage in -70°C freezer for 4 days
T4	Storage in freezer(s) -30 °C for 1 day, -50 °C for 1day, -70 °C for 1 day and plunge into liquid nitrogen (-196 °C) for 1 day
T5	Storage in freezer(s) -30 °C for 1 day, -50 °C for 1 day and plunge into liquid nitrogen (-196 °C) for 2 days
T6	Storage in -50 °C freezer for 2 days and plunge into liquid nitrogen (-196 °C) for 2 days

Table 2.1. Describes design for all six treatments

2.2.5. Determination of cooling rates for six treatments

Grant squirrel data logger (1200 series) was used in determining cooling rates for all six treatments. Three seeds were chosen free of cracks, for each treatment and individual seed drilled using high speed metabo twist drill to create hole not more than 1 mm. Type k thermocouples were inserted into each seed and the hole sealed with adhesive bathroom sealer before the seed was placed in its respective aluminium foil bag. Three replicates were used in determining cooling rates for each treatment comprising of one seed in its respective foil bag, folded and clipped with office pin; all totaling three seeds in three aluminium foil bags and an empty foil bag to determine difference in cooling rate between seed and bag and bare type k thermocouple from the data logger serving as the control for each treatment.

Temperature was measured and stored by data logger every 15 seconds and the cooling rates interpolated from print-outs of the treatment runs using the steepest part of the temperature reduction curve, which varied considerably between treatments. Data in this study was presented as mean maximum cooling rate.

The relevant references for the six cooling treatments applied to *P.pinea* seeds are shown on table 2.2.

TREATMENT	DETERMINED COOLING RATES (°C/min)	REFERENCES
T1 (-196 °C for 4 days)	165.0	Pita *et al.*, 1998
T2 (-50 °C for 4 days)	13.8	Dussert *et al.*, 2001
T3 (-70 °C for 4 days)	17.8	This study
T4 (-30 °C for 1 day; -50 °C for 1 day; -70 °C for 1 day and -196 °C for 1 day)	11.3 13.8 17.8 107.0	Dussert *et al.*, 2001
T5 (-30 °C for 1 day; -50 °C for 1 day; and -196 °C for 2 days)	11.3 4.0 111.0	This study
T6 (-50°C for 2 days and -196°C for 2days)	14.5 140.0	This study

Table 2.2 shows the mean maximum cooling rates of *P. pinea* seeds for the six cooling treatments.

- It was observed from data presented, that cooling rate was slower than that reported for seeds of other species put into liquid nitrogen which can exceed 200° C/min. The relatively slow cooling rate in *P. pinea* seeds was probably due to the large size of the seeds, which slowed down temperature flow.

2.2.6. Preparation of 1 % water agar

20 g of agar was measured on a balance and mixed with 200 ml cold distilled or deionised water in a stainless steel jug and this solution poured into another stainless jug with 1800 ml of distilled water. The solution was brought to boil on a hot plate.

Heating was stopped when bubbles were observed and the solution started to rise. The agar was allowed to cool for 15 - 20 minutes, and about 5 ml of agar was poured into individual sandwich boxes and allowed to cool for 30 minutes before storage in a refrigerator at 5 °C until use.

2.2.7. Methodology for conductivity tests

Conductivity tests were carried out to determine whether cryopreservation stress resulted in increased electrolyte leakage i.e. to test whether membranes are damaged by the cryopreservation protocol. To conduct this experiment the CM100, conductivity measurer (Reid, South Africa) was used. The CM 100, conductivity measurer measures the conductivity of water which is influenced by the amount of electrolyte leakage through broken membranes.

The procedure for this experiment was as follow:

(i) Nine individual seeds of *P.pinea* and *P.caribaea* were selected carefully on the basis that they were free from cracks or punctures.

(ii) Each seed and its individual well are labelled 1 - 9 for identification.

(iii) Fresh weights of the individual nine seeds of the samples were taken one after the other in both species.

(iv) After which wells were filled with 0.5 ml deionised water per well for a total of 18 individual wells per test by pipetting and transferring deionised water from laboratory beaker to conductivity measurer (CM 100) wells .

(v) Each labelled seed was placed in its corresponding labelled well.

(vi) Thereafter, the wells were placed in a conductivity meter (CM100) and programmed under the control of its computer for a set time (i.e. 48 hours) and readings were recorded every 30 mins by conductivity measurer (CM 100) into database system of computer.

(vii) After measurement, each individual seed was blotted dry with 2-ply tissue paper and reweighed.

(viii) All individual seeds were placed in dry weight dishes labeled correspondingly to the individual seeds and placed in the oven at 103 $^{\circ}$C for 17 hours to evaporate the water.

(ix) Individual seeds were reweighed to get the dry weight.

(x) Seed conductivity/per dry weight seed ($\mu Sgdw^{-1}$) was calculated.

* Note that conductivity measurements were taken at a controlled room temperature of 20° C and the voltage of the conductivity measurer was held at two volts.

2.3. Extended storage tests

The purpose of this experiment was to determine whether longer periods of storage of seeds in liquid nitrogen would affect their vigour and germination.

Pinus caribaea seeds were stored in liquid nitrogen for 2, 4, 8, 12, 16, 20, 24, 28 days; each treatment comprising of seeds in their aluminium packets was taken out immediately within the time span of 1 minute and placed on laboratory bench to thaw for 24 hours followed by germination for 28 days.

The control for this experiment was seeds sown on agar for 28 days; held at 20 °C room temperature.

2.3.1. Survival studies of *P. pinea* seeds equilibrated to different moisture contents prior to storage and sowing

Survival studies were conducted to determine at which moisture content seeds of *P. pinea* showed stress in their germination.

Seeds of *P.pinea* were equilibrated to 5 %, 7 %, 9 %, 12 % and 16% moisture contents (fw) by putting sets of 85 *P.pinea* seeds on cover of sandwich boxes and placing in equilibration chambers filled half way with prepared solutions of LiCl salts of weights 5 g, 10 g, 15 g, 20g, 25 g, 30 g, 40 g, 50 g, 60 g, 70 g, 80 g, 90 g per 100ml of deionised water, and one chamber with and silca gel alone .

prepared solutions of LiCl salts of weights 5 g, 10 g, 15 g, 20g, 25 g, 30 g, 40 g, 50 g, 60 g, 70 g, 80 g, 90 g per 100ml of deionised water, and one chamber with and silca gel alone .

Seeds were removed from each equilibration chamber for regular weighing (daily basis) until 'constant' weights were achieved per seed treatment. Then five seeds out of 85 seeds were placed on rotronic hygrometer to determine relative humidities to establish approximated moisture contents (Personal communication; John Adams, Royal Botanic Gardens, Millenium Seed Bank, Wakehurst Place, UK). Moisture contents determined at the end of the equilibration period were 5 %, 7 %, 9 %, 12 % and 16 % (see Table 3.4.b).

All seeds were equilibrated at controlled room temperature of 20 °C; prior to direct plunge into liquid nitrogen and sowing on agar for 28 days.

2.3.2. Differential scanning calorimetry tests

Differential scanning calorimetry tests were carried out with a view to determine at what temperatures lipids and water melt or solidify.

A DSC model 7 (Perkin Elmer, UK) was used in this test to determine melting and solidification transitions of isolated or excised whole embryos of *P. pinea*. In general the differential scanning calorimeter allows calorimetric measurement, characterization and analysis of the thermal properties of materials. Then under the control of a computer the differential scanning calorimeter was programmed from an initial to final temperature and measures the ensuing thermal transitions in the sample material. The cryopreservation laboratory was close to research laboratory for the purpose

of assessing coolant (liquid nitrogen), purge gas (helium) and pump gas (nitrogen).

DSC aluminium pans and lids were weighed on an ultramicrobalance (Sartorius 4504 MP-1, precision=± 0.1 μg) to which about 5 mg of tissue was added.

Forty *P. pinea* seeds were selected and their embryos excised from the female gametophyte and seed coat; sterilizing all 40 embryos with 0.5 ml sodium hypochlorite (NaOCl); rinsing with deionised water and equilibrating them with lithium chloride salts (LiCl) to the respective moisture contents (fw) of 5 %, 7 %, 9 %, 11 %, 13 %, 15 % and 18 % while seeds equilibrated to 26 % with water.

Excised embryos were chopped into 5 parts, not including the cotyledons and each sample was transferred to the bottom of aluminium pan. The pan lid was put in place and sealed using a crimper and each sample was placed in the chamber of the machine using the suction 'pooter'. The chamber guard was used to prevent the sample pan from falling into the machine, then the lid of the sample chamber was replaced and main chamber swing-lid was closed to allow equipment to start running .

The differential scanning calorimeter was calibrated before experiment on samples using the melting of indium (melting point, 156.60°C; transition energy, 28.45 J/g). The thermal lag of the calorimeter was checked using the melting point of double deionised water.

The experimental procedure for determining melting or solidification transitions in each seed sample was programmed on computer as follows:

(i) Cool from 20 °C to -100 °C at 10 °C/min;

(ii) Hold at -100 °C for 1 min (to allow the sample to reach thermal equilibrium);

(iii) Warm from -100 °C to 50 °C at 10 °C/min.

(iv) Cool to 20 °C.

The total amount of melting (water and lipids) was calculated from the area under the energy curve (endotherm) during warming using the Pyris Thermal Analysis software.

It should be noted that operation of this equipment was supervised by trained and authorized laboratory personnel.

2.3.3. Statistical analysis

In germination tests statistical analysis was conducted using Minitab software (version 13.1) to carry out the Tukey's pairwise comparison test. At confidence level $P < 0.05$ same letters are not significantly different from one another.

In both conductivity and extended storage studies statistical analysis was completed using the Statistical Package for Social Sciences (SPSS) to carry out one – way Analysis of Variance (ANOVA) were same letters are not significantly different from one another at confidence level of $P < 0.05$.

CHAPTER THREE

RESULTS

3.0. Introduction

In this chapter the results are presented on germination, before and after cryo-storage, using six treatments. Changes in seed leakage, following cryo-storage and differential scanning calorimetry are also reported. Experiments on germination before and after storage at different temperatures were conducted with a view to designing a new cryo-protocol for the effective storage of pine seeds and improving the protocol of Pita *et al.* (1998) which showed viability loss in germination after short term storage in liquid nitrogen. Conductivity experiments were conducted to test leakage in two of the three species (*P. pinea* and *P. caribaea*) using CM100 conductivity meter to determine total leakage rate as a measure of changed vigour following low temperature storage.

The results from these studies are reported, analysed and discussed.

3.1. Germination of three pines after six different storage treatments

The three pines were initially sown on agar in sandwich boxes as controls for the subsequent storage treatments for *P. pinea, P. pseudostrobus* and *P. caribaea* which had 4 replicates of 20 seeds, totaling 80 seeds per species. This allowed a comparison with the germination after the six different treatments.

The results of germination are given in Table 3.1 and show differences between controls and treatments stored at low temperatures.

Table 3.1. Germination percentage for three *Pinus* species stored at six different treatments compared to the control

Species	Treatment	Germination (%) (with standard deviation)
Pinus pinea	control	93.75 ± 7.50[b]
	-196 °C (4 days) [T1]	98.75 ± 2.50[a]
	-50 °C (4 days) [T2]	87.50 ± 5.00[c]
	-70 °C (4 days) [T3]	87.50 ± 10.41[c]
	-30 °C (1 day)+ -50 °C (1 day)+ -70 °C (1 day)+ -196 °C (1 day) [T4]	97.75 ± 2.50[a]
	-30 °C (1day)+ -50 °C (1 day)+ -196 °C (2 days) [T5]	97.50 ± 2.89[a]
	-50 °C(2 days) + -196 °C (2days) [T6]	100 ± 0.00[a]
Pinus pseudostrobus	control	48.75 ±6.29[ab]
	-196 °C(4days) [T1]	40.00 ± 10.80[b]
	-50 °C(4days) [T2]	53.75 ± 13.15[a]
	-70 °C(4days) [T3]	42.50 ± 8.66[b]
	-30 °C(1day) + -50 °C (1day)+ -70 °C (1day) + -196 °C(1day) [T4]	50.00 ± 9.13[ab]
	-30 °C(1day) + -50 °C (1day)+-196 °C (2days) [T5]	51.25 ± 17.97[a]
	-50 °C (2days) + -196 °C(2days) [T6]	52.50 ± 5.00[a]
Pinus caribaea	Control	66.25 ± 9.46[a]
	-196 °C(4days) [T1]	60.00 ± 10.80[ab]
	-50 °C(4days) [T2]	60.00 ± 9.13[ab]
	-70 °C(4days) [T3]	66.25 ± 23.94[a]
	-30 °C(1day) + -50 °C (1day)+ -70 °C (1day) + -196 °C(1day) [T4]	67.50 ± 6.45[a]
	-30 °C(1day) + -50 °C (1day)+-196 °C (2days) [T5]	53.75 ± 11.09[b]
	-50 °C (2days) + -196 °C(2days) [T6]	52.50 ± 5.00[b]

*figures with same letters are not significantly different at $p<0.05$ using Tukey's pairwise comparison.

For *P. pinea* seeds, T1, T4, T5 and T6 had the highest recovery levels following cryopreservation, while the lowest recovery level was seen in both T2 and T3. A comparison of T6 with T1, T4 and T5 revealed no significant differences, there was significant difference between the control and six treatments.

Whilst T6 showed value of 100 ± 0.00 %, the control showed a mean value of 93.75 ± 7.50 %. In comparing all six treatments with the control the least suitable protocols to adopt were T2 and T3 for the storage and preservation of *P. pinea* seeds.

In the case of *P. pseudostrobus* seeds, no significant difference was seen between its control and all six treatments, but interestingly significant differences were observed among treatments with the exception of T4 which is not significantly different from other five treatments. T2, T5 and T6 are significantly different from T1 and T3. From all indications T1 and T3 are the least suitable protocols to adopt in the storage and preservation of *P.pseudostrobus* seeds.

In *P. caribaea* seeds, T1, T2, T3 and T4 were observed to have the highest recovery levels, and no significant differences were observed between its control and T1, T2, T3 and T4. While the least suitable treatments T5, T6 showed significant difference from the control and T3, T4.

The three pines had protocols or treatments suited for their germination, namely;

- *P. pinea* – T1, T4, T5 and T6
- *P. pseudostrobus* – T2, T4, T5 and T6
- *P. caribaea*- T1, T2, T3 and T4.

- Statistically treatments shown as suited for cryopreservation in *P.pinea* and *P.caribaea* above have numerical trends to be checked upon by doing future work on them.

Among all three species *P.pinea* seeds had the highest germination levels, before and after cryopreservation.

Fig.3.1.

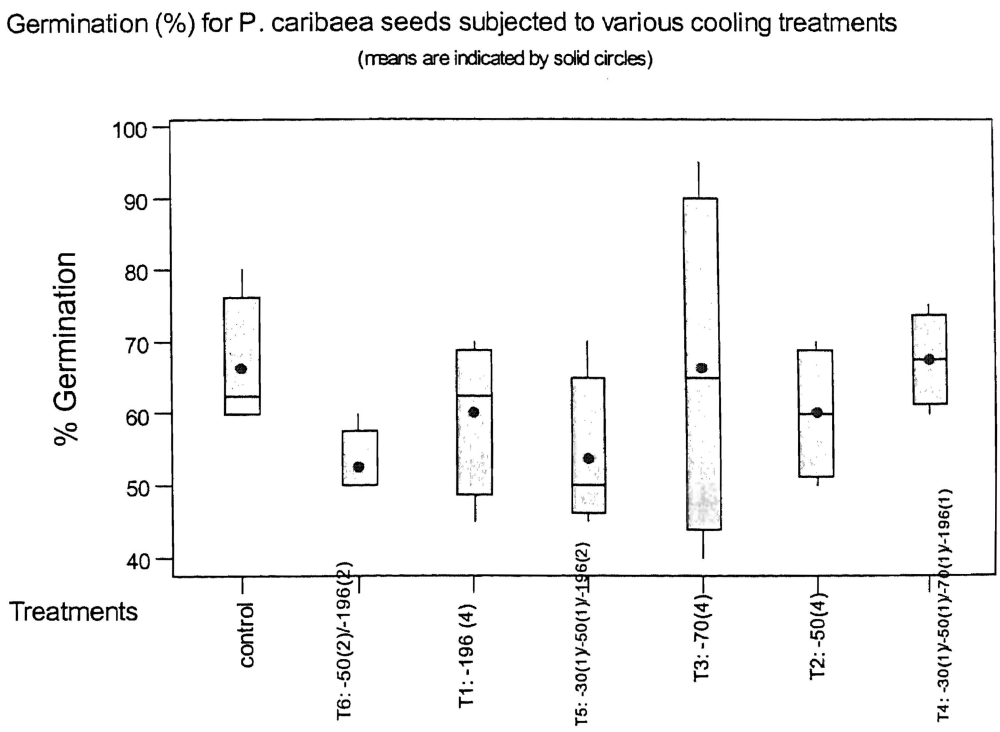


Fig.3.2.

Germination (%) for P. pinea seeds subjected to various cooling treatments
(means are indicated by solid circles)

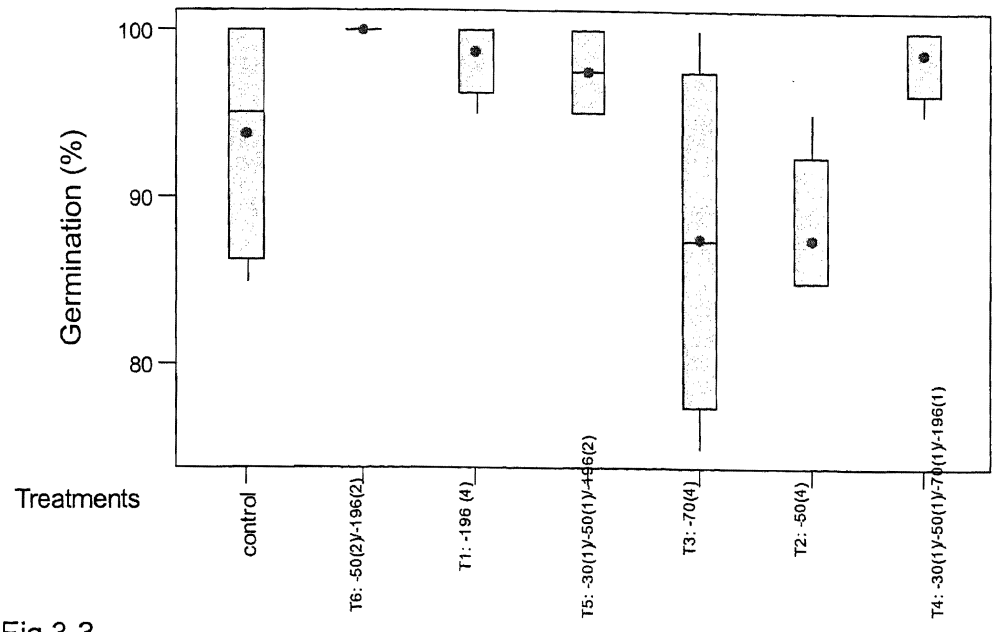
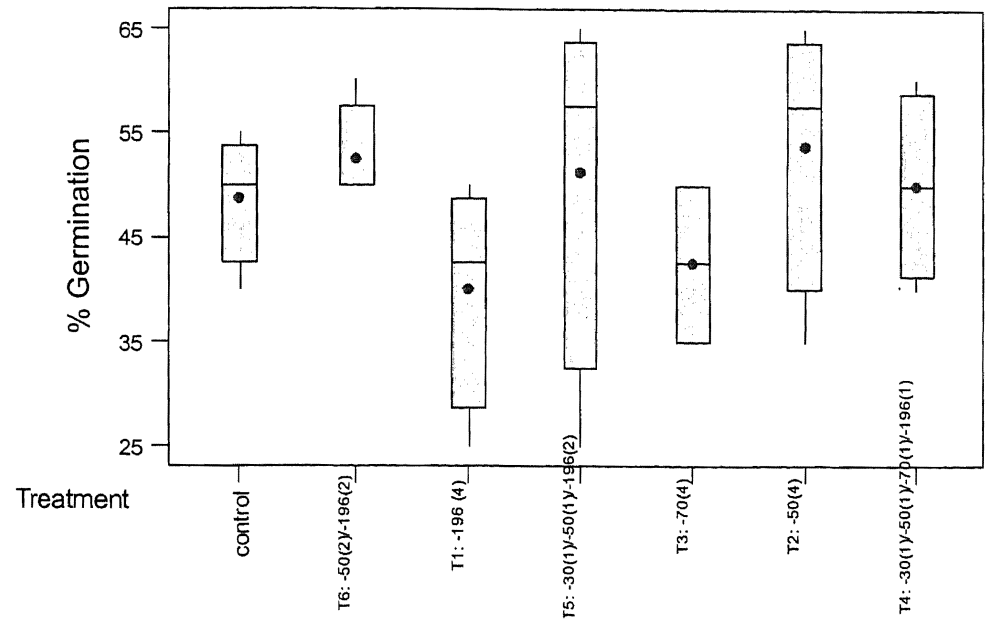


Fig.3.3.

Germination (%) for P. pseudostrobus seeds subjected to various cooling treatments
(means are indicated by solid circles)



Note that solid circles represents mean values while the bars represents the median values.

3.1.1. Progressive profile germination

Germination peak periods were determined using germination progressive curves to visualize rates for each species of pine germination; Figs. 3.4, 3.4.1, 3.5, 3.5.1, 3.6 and 3.6.1. The purpose of this study is to plot germination progressive curves and assess visually, to see if there were any gross changes in germination rates as a result of the applied treatments.

The control set of *P. pinea* seeds showed a lag period of 8 days during which there was no visible germination of seeds. The majority of seeds germinated during the period 9^{th} - 20^{th} day; from the 21^{st} - 28^{th} day there was little or no germination. At the end of the test period percentage germination in *P. pinea* seeds was 93.75 % (fig.3.4). However, in the control of *P.pseudostrobus* seeds, there was no visible for the first seven days and most germination occurred in the 8^{th} - 20^{th} days, after which there was little or no germination recorded. After 28 days percentage germination was 48.75% (Fig.3.5). Control of *P.caribaea* seeds showed a similar pattern of germination; from 0-7^{th} day no seeds germinated, germination followed on the 8^{th} - 20^{th} day, but from the 22^{nd} - 28^{th} day there was little or no change in germination (Fig.3.6).

T1 showed highest germination in *P. pinea* seeds, while lowest percentage rates were seen in T2 and T3. After the lag period of 9 days, T1 had the highest germination rate (Fig. 3.4.1), in contrast with T2 and T3 whose rates were visibly lower following a similar lag period. T6 had percentage germination of 100 % and T1 98.75 % after the 28^{th} day, but did not show the

uniformly high level of germination seen with T1. In comparison with the control (Fig.3.4) T1 which

was observed to have the highest rate of germination, showed more uniformity and higher level in germination of seeds than in the control.

In contrast with *P. pinea* seeds, *P. pseudostrobus* seeds showed highest germination with T5 and T6, while T1 and T3 were seen to germinate more slowly. After 28 days of germination, T5 and T6 had 51.25 % and 52.50 % respectively, while T2 at the end of lag period had highest percentage germination. Comparing this with the control, T5 and T6 showed higher frequency and uniformity in germination level, rather than of the control which showed a scattered and unstable rate of germination in the 9^{th} - 20^{th} days.

* Figs 3.4 to 3.6 show the impact of the different cryo-treatments on the germination profile of the three pine species.

In *P. caribaea* seeds T6 was observed to have the highest germination, while T3 was observed to have the least percentage germination during the period of 18 - 20^{th} day (Fig.3.6.1). T6 had percentage germination of 52.50 % after 28 days. Comparing control of *P. caribaea* seeds with the two other pines, the rate at which it germinated was higher *than P.pinea* which had the highest rate of germination during 9^{th}-20^{th} day period.

Generally, from all three species of pine (*P. pinea, P. pseudostrobus, P. caribaea*) the first nine days were a lag period in which little or no germination is visible, while from the 9^{th} day - 20^{th} day there normally was increased germination and at 20^{th} day to the 28^{th} day there was little or no further germination. Graphically, gross differences were not seen except clearly for

two treatments in both *P. pinea* (T2 and T3) and *P.pseudostrobus* (T1 and T3), but no visible difference in *P.caribaea* seeds. From all observations made in this study it showed that lower germinating seed-lots tends to be slower when germinating while high germinating seed-lot vice-versa. For example in aubergine seeds (*Solanum melongena*) four seed-lots were compared. The first seed-lot which had 93 % seedling emergence germinated at a mean emergence time of 2.6 days; second seed-lot showing 78 % seedling emergence germinated at a mean emergence time of 3 days; third seed-lot with 73 % seedling emergence germinated also germinated 3 days of mean emergence time and fourth seed-lot with 63 % seedling emergence germinated at a mean emergence time of 4.3 days (Demir *et al.,* 2005).

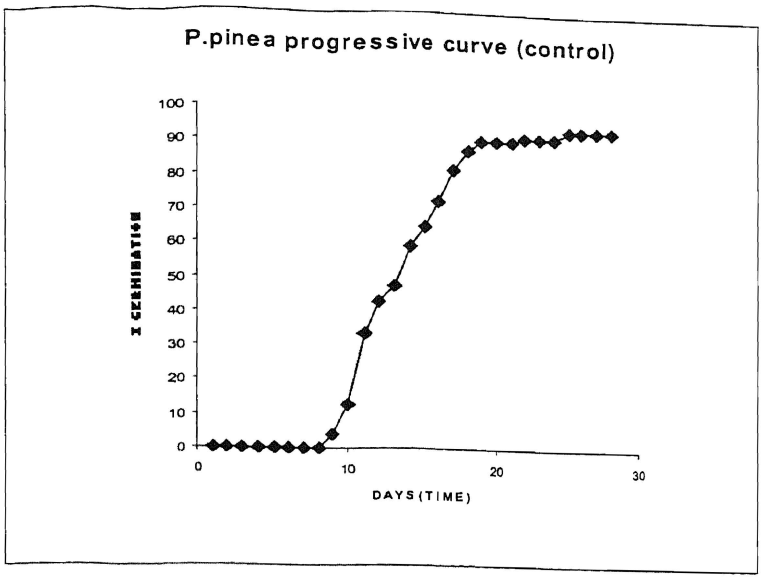


Fig. 3.4. Percentage germination rates in the control of *P. pinea* seeds.

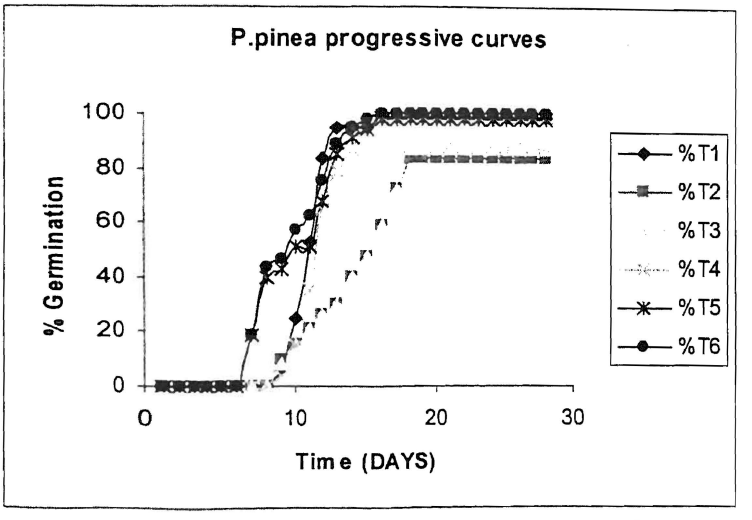


Fig. 3.4.1. Percentage germination rates of six treatments for *P. pinea* seeds (See Table.3.0 for description of each treatment).

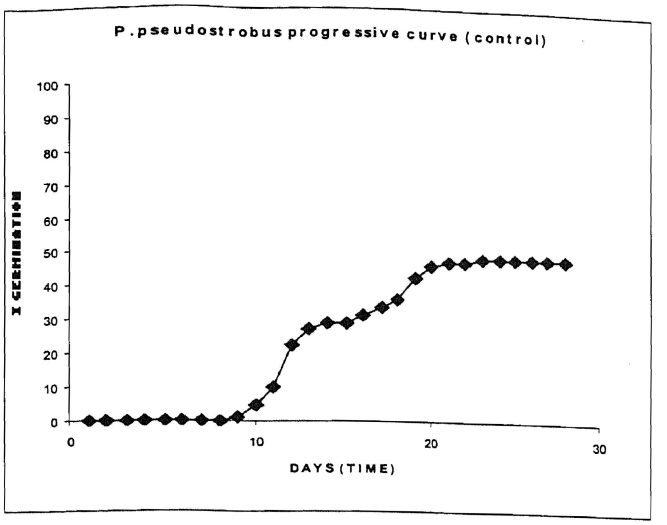


Fig.3.5. Percentage germination rates of *P. pseudostrobus* seeds.

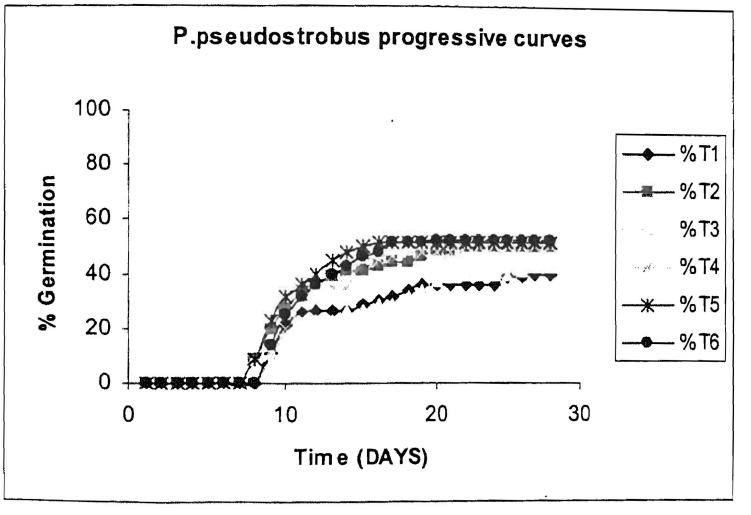


Fig 3.5.1. Percentage germination rates of six treatments for *P. pseudostrobus* seeds (See Table.3.0. for description of each treatment).

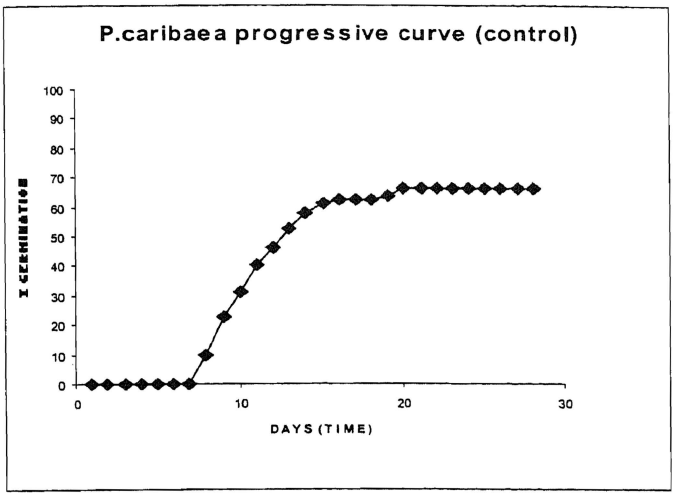


Fig.3.6. Percentage germination rates of *P. caribaea* seeds.

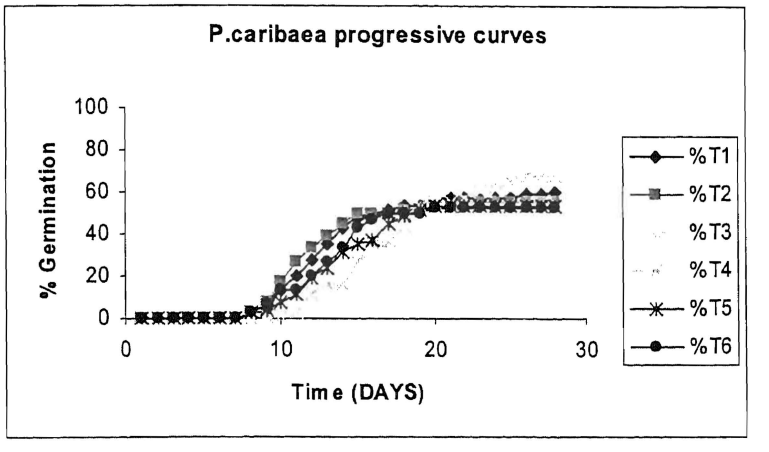


Fig.3.6.1. Percentage germination rates of six treatments for *P.caribaea* (See Table.3.0. for description of each treatment).

3.2. Measurement of total leakage

P. pinea and *P. caribaea* seeds were selected for a comparison of seed leakage, as the two species differed markedly in their response to cryo treatments T6, where *P.caribaea* had a large fall in viability compared to *P.pinea;* and also in respect T2 and T3.

Nine seeds of each species were carefully selected from their seed-lots; checked as free of cracks and labeled, before being placed in CM100 conductivity meter for 48 hours. All six treatments used for storage were assessed by conductivity (Figs 3.7 and 3.8).

Conductivity measurements showed that *P. pinea* seeds following T2 treatment showed the highest total leakage of 427.1 $\mu Sgdw^{-1}$ ± 5.9 (Table.3.2., Fig.3.7) and significantly different from other treatments and its control, whereas, in *P.caribaea* seeds T1 showed highest total leakage of 2254 $\mu Sgdw^{-1}$ ± 30.5 (Table. 3.3., Fig. 3.7.1) and significantly different from other treatments and its control.

The results suggest that leakage of *P. caribaea* seeds is not a marker of stress in these seeds. T1 showed highest leakage rate (Fig. 3.7.1) as compared with T6 the lowest percentage germination and lowest leakage level. However, leakage in *P. pinea* seeds might be a marker of stress, because T2 showed highest leakage rate and the lowest percentage germination.

All treatments were taken and recorded, although low levels in treatments T3, T4, T5 and T6 can not be explained (figs. 3.7 and 3.7.1).

Table.3.2. Leakage in *P.pinea* ($\mu Sgdw^{-1}$)

TREATMENTS	Mean conductivity ± Standard error
CONTROL	$151.5^{a} \pm 3.2$
T1	$325.9^{b} \pm 2.6$
T2	$427.1^{c} \pm 5.9$
T3	$235.7^{de} \pm 5.9$
T4	$199.2^{d} \pm 4.5$
T5	$221.8^{e} \pm 1.6$
T6	$228.6^{e} \pm 2.4$

* Figures with the same letters are not significantly different at $p < 0.05$ using one way anova comparison.

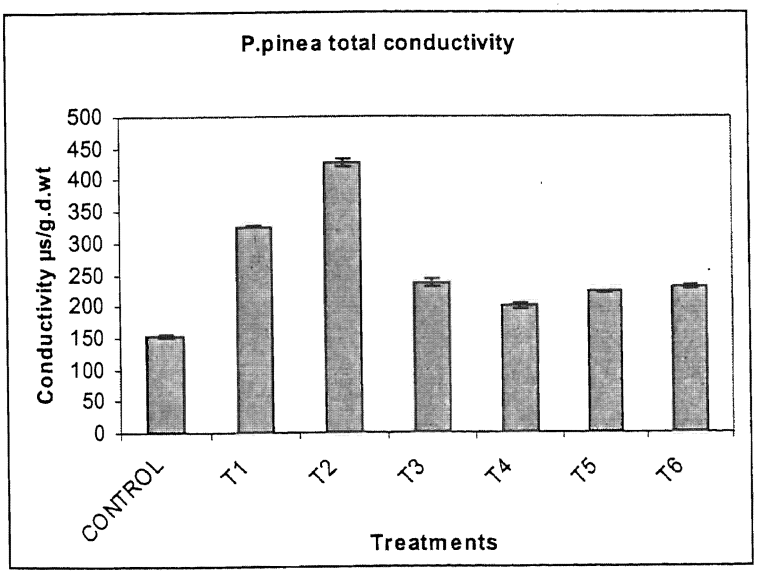


Fig 3.7. Total leakage in *P. pinea* whole seeds after 48 hours in all six treatments compared with control all in measured values of µS/g.d.wt.

Table.3.3. Leakage in *P.caribaea* ($\mu Sgdw^{-1}$)

TREATMENTS	Mean conductivity ± Standard error
CONTROL	$2091^{a} \pm 46.5$
T1	$2254.2^{b} \pm 30.5$
T2	$1001.8^{c} \pm 49.9$
T3	$660.9^{de} \pm 44.2$
T4	$640.7^{de} \pm 20.7$
T5	$738.7^{d} \pm 10.1$
T6	$580.3^{e} \pm 3.0$

* Figures with the same letters are not significantly different at $p < 0.05$ using one way anova comparison.

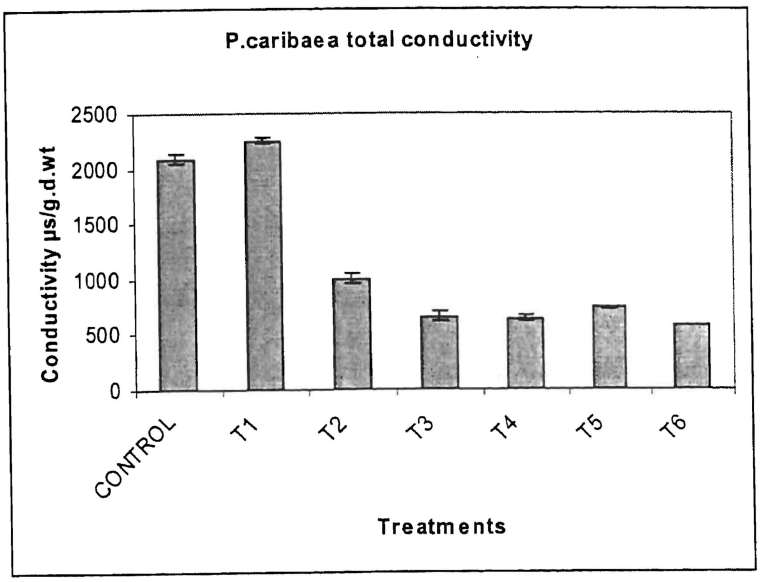


Fig 3.7.1. Total conductivity in *P. caribaea* whole seeds after 48 hours in all six treatments compared with control all in measured values of µS/g.d.wt.

3.3. Effect of extended storage of *P. caribaea* seeds in liquid nitrogen

Following the conductivity studies on the two selected seed species, namely *P. pinea* and *P. caribaea, P. caribaea* seeds were placed in liquid nitrogen for eight different time periods, namely - 2, 4, 8, 12, 16, 20, 24 and 28 days. eighty seeds were selected for each treatment, i.e. 20 seeds in each of 4 replicates.

Seeds were placed in aluminium hermetically sealed bags and placed in Nalgene boxes with the right order; i.e. from seeds replicates of 2 days to 28 days.

However, seeds when taken from Nalgene boxes, were not allowed to thaw because time of removal from liquid nitrogen cryotanks and replacing those samples of seeds back into liquid nitrogen was very short (in 8 - 10 seconds) thus reducing the risk of cycling effect of the eight different times seeds were taken out of liquid nitrogen and plunged back.

Survival studies on *P. caribaea* were undertaken to find out whether or not the germination level of this pine can be affected by extended storage time in liquid nitrogen.

However, percentage germination taken from all eight storage times in this study showed that storage in liquid nitrogen (-196 °C) for longer than 4 days resulted in a gradual decrease in the percentage germination (Fig 3.8). As seeds were stored longer in liquid nitrogen there was a gradual decline in the percentage germination (Table. 3.4).

However, when comparing the control with the storage times of 2 to 24 days there were no significant differences with the exemption of the 28th day which showed significant difference from all other treatments, thus explaining the fact that storing seeds of this specie (*P. caribaea*) for 28 days or more in liquid nitrogen may be detrimental (Table.3.4 and fig.3.8).

Days (immersed in liquid nitrogen)	Germination (%) ± standard error
control	66.3 ± 9.5^{a}
2	73.7 ± 3.8^{a}
4	65.0 ± 5.4^{a}
8	62.5 ± 11.3^{a}
12	60.0 ± 4.1^{a}
16	60.0 ± 4.6^{a}
20	61.3 ± 4.3^{a}
24	56.3 ± 3.8^{a}
28	51.3 ± 8.0^{b}

Table. 3.4. The percentage germination achieved after immersing or plunging *P. caribaea* seeds into liquid nitrogen (-196 °C) for different intervals prior to sowing on agar for 28 days.

- Figures with the same letters are not significantly different at $P< 0.05$ using one way anova comparison.

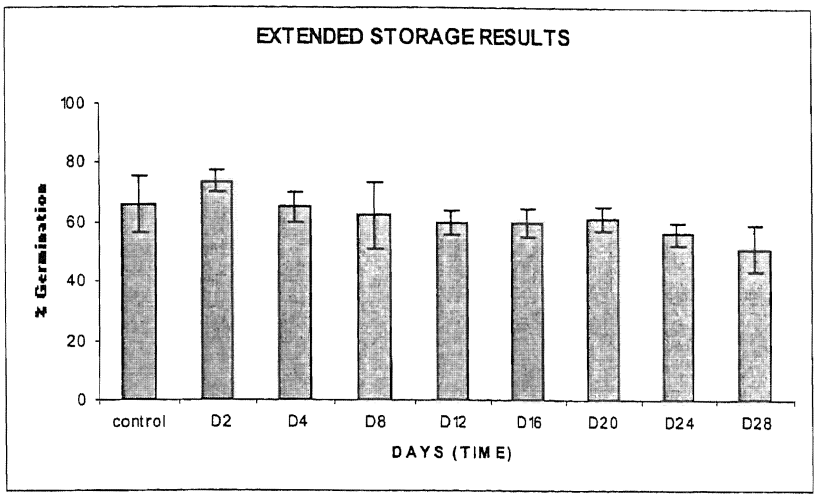


Fig.3.8 Percentage germination rates after storage of *P. caribaea* seeds in liquid nitrogen days prior to sowing on agar for 28 days.

3.4. Effects of different moisture contents in storage of *P. pinea* seeds

Experiments were conducted to determine whether the moisture content of *P. pinea* seeds was a problem when Pita *et al.* (1998) carried out their research on seven native Spanish species of pine and showed a decrease in germination of seeds after storage in -196°C for 4 days. According to Stanwood (1985) the critical water content for freezing injury in orthodox seeds varies between 10-30 %. In the study by Pita *et al.*(1998), the *P. pinea* seeds used had 9.82 % moisture content which could be close to the critical water content for an oily seed.

To conduct this study 85 *P.pinea* seeds were placed on sandwich box cover for each equilibration chamber containing LiCl salts with weights (in grams) of 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90 and silica gel. Then seeds were weighed daily until constant weight was achieved in each equilibration

chamber after which five seeds from each equilibration chamber were picked and their relative humidities measured on rotronic hygrometer. The exact moisture contents of *P.pinea* seeds at different relative humidities at (controlled room temperature) 20 °C are shown in Table 3.4b.

Moisture contents were plotted against relative humidities, enabling the presentation of an isotherm to determine moisture content (fw) of seeds used in this study (Fig 3.9). Seeds equilibrated to 5, 7, 9, 12 and 16 percent moisture content (fw) with corresponding percentage relative humidities of 20, 50, 64 and 90 approximately were stored in liquid nitrogen for four days prior to sowing on agar for 28 days to germinate. The results fig 3.9.1, indicate that at 12 % moisture content (fw) there was a reduction in germination of seeds after storage suggesting that ice formation starts between 12 - 16 % moisture content (fw); that is – to say that even at 10 % moisture content (fw) the effect of freezing is not lethal in *P. pinea* seeds. This suggests that the critical moisture content can not be used to explain the reduction in germination of seeds in *P. pinea* seeds reported by Pita *et al.* (1998) or the effects of T2 and T3 on dry seeds of *P. pinea*.

The method used in this study was robust and the measured seed relative humidity percentage was very close to the expected seed relative humidity percentage (Personal communication; John Adams, Royal Botanical Gardens, Millennium Seed Bank, Wakehurst Place, UK) for the various LiCl salt concentrations (Table 3.4. b).

LiCl salts (in grams)	Moisture content (fw) (%)	Measured seed relative humidity (%)	Expected seed relative humidity (%)
5	18.8	92.0	95.0
10	13.0	90.0	88.0
15	12.0	81.0	83.0
20	11.2	84.5	79.0
25	10.0	79.8	75.0
30	8.0	64.0	60.0
40	7.0	50.0	44.0
50	6.5	40.0	33.0
60	6.2	30.0	24.0
70	5.0	20.0	18.0
80	4.9	18.8	13.0
90	4.7	10.8	11.0
Silica gel	3.0	9.8	9.0

Table 3.4. shows measured seed relative humidity, expected seed relative humidity percentages with their corresponding percentage moisture contents (fw).

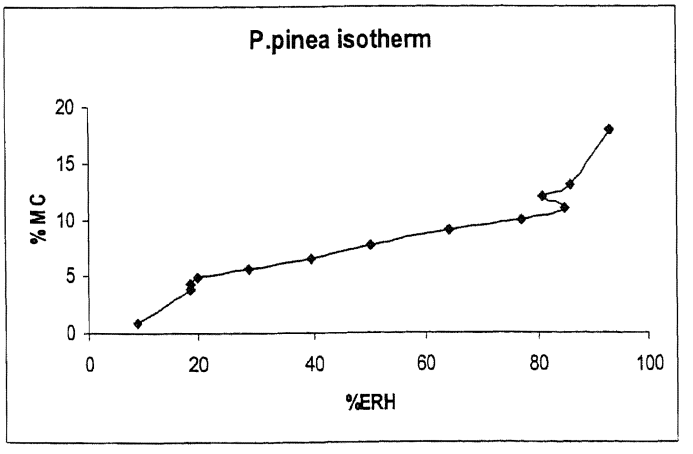


Fig 3.9. Isotherm of *P. pinea* whole seeds after being equilibrated on LiCl salts at 20 °C dry room temperature.

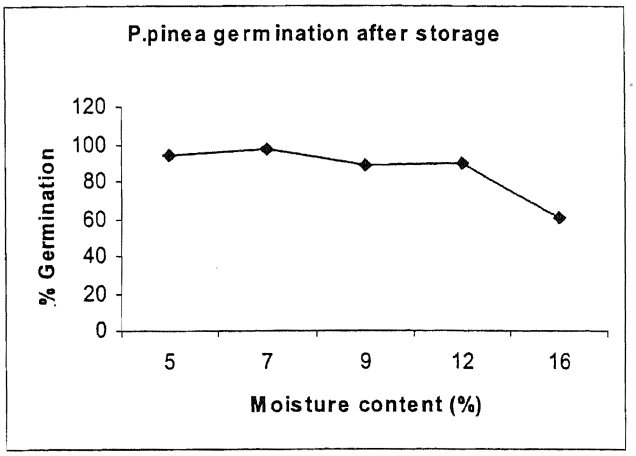


Fig 3.9.1 *P. pinea* seeds percentage germination after storage in liquid nitrogen for four days prior to sowing for 28 days with five different moisture contents.

3.5. Determination of melting transitions using differential scanning calorimetry in *P. pinea* embryos

To determine whether lipids (Vertucci,1989) were the problem associated with the reduced germination of *P. pinea* seeds after cryopreservation (Pita *et al.,* 1998) five embryos of *P. pinea* seeds were taken for each of these respective moisture contents (fw)- 5, 7, 9, 11, 13, 15, 18 and 26 % and analysed on the differential scanning calorimeter using the Pyris thermal analysis to determine heat flow during cooling and heating cycles.

In comparing the first six cooling thermograms- 5, 7, 9, 11, 13 and 15 % moisture content (fw) with the last two (18 and 26 % MC) it was seen that ice formation did not occur in the first six moisture contents (fw) (5, 7, 9, 11, 13 and 15 %) but noticeable in the last two moisture contents (fw) (18 and 26 %).

Furthermore, in the first four moisture contents (fw) it was noticed that at 5, 7, 9, 11 % glass transitions could be seen (Figs 3.9.2, 3.9.3, 3.9.4 and 3.9.5). In the last 2 moisture contents there was ice formed at temperatures between -8 and -14 °C.

However, heating cycles were also analysed to characterise transitions in pine embryos. Observing from thermograms for tissue at moisture contents of 5, 7, 9, 11 and 13% moisture content (fw) showed the presence of lipid transitions (Fig. 4.0, 4.1, 4.2, 4.3 and 4.4). Only at 15 % MC did the thermogram shows two small peaks, indicating that water and lipids interacting with one another and probably the presence of ice crystals. Dussert *et al.,* 2001 explained the fact that the interaction between lipids and water can form small pernicious ice that can grow into larger ones which can be lethal. Furthermore, heat cycles of *P.pinea* embryos with 18% and 26% moisture content (fw) indicated that ice was recrystallized at temperature of -20°C, hence, suggesting that ice crystals lethal to seeds are formed at two moisture contents stated above (Fig. 4.5, 4.6 and 4.7).

Furthermore, three distinctive peaks were observed in the cooling thermograms of 5, 7, 9, 11, 13% but thermogram with fig.3.9.7 of 15%

moisture content (fw) showed two distinctive peaks which indicated the prescence of water and lipids transitions at -18°C and -28°C and thermograms of 18 and 26% moisture content (fw) showed only one noticeable peak; while with heating thermograms two distinctive peaks were noticed with also the exception of 18% and 26% moisture content (fw) showing shifts in their temperatures which might be due to impurities in sample of embryos (Table.3.4.c and Table.3.4.d).

The above data shows how heat flow was dissipated at the various moisture contents demonstrating that at moisture contents between 15% moisture content (fw) and 26% moisture content (fw) more energy was used up, showing that ice was recrystallized and suggesting that at moisture contents between 15 and 26% moisture content (fw) ice crystals are formed and are lethal to the viability of *P.pinea* seeds.

Moisture contents (%)	Peak 1 (Onset temperature, ° C)	Peak 2 (Onset temperature, ° C)	Peak 3 (Onset temperature, ° C)
5	-20	-25	-35
7	-20	-25	-35
9	-22	-28	-38
11	-22	-28	-38
13	No observation of peak	-25	-40
15	-18	-28	-45
18	No observation of peak	-15	No observation of peak
26	No observation of peak	-8	No observation of peak

Table. 3.4.c. Temperatures at which peaks are formed at cooling cycle of *P. pinea* embryos at different moisture contents.

Moisture content (%)	Peak 1 (Onset temperature, ° C)	Peak 2 (Onset temperature, ° C)	Peak 3 (Onset temperature, ° C)
5	-18	-30	No observation of peak
7	-18	-30	No observation of peak
9	-18	-30	No observation of peak
11	-18	-30	No observation of peak
13	-18	-28	No observation of peak
15	-10	-20	No observation of peak
18	-18	-5	5
26	-18	-5	5

Table. 3.4.d. Onset temperatures at which peaks are formed at heating cycles of *P. pinea* embryos at different moisture contents.

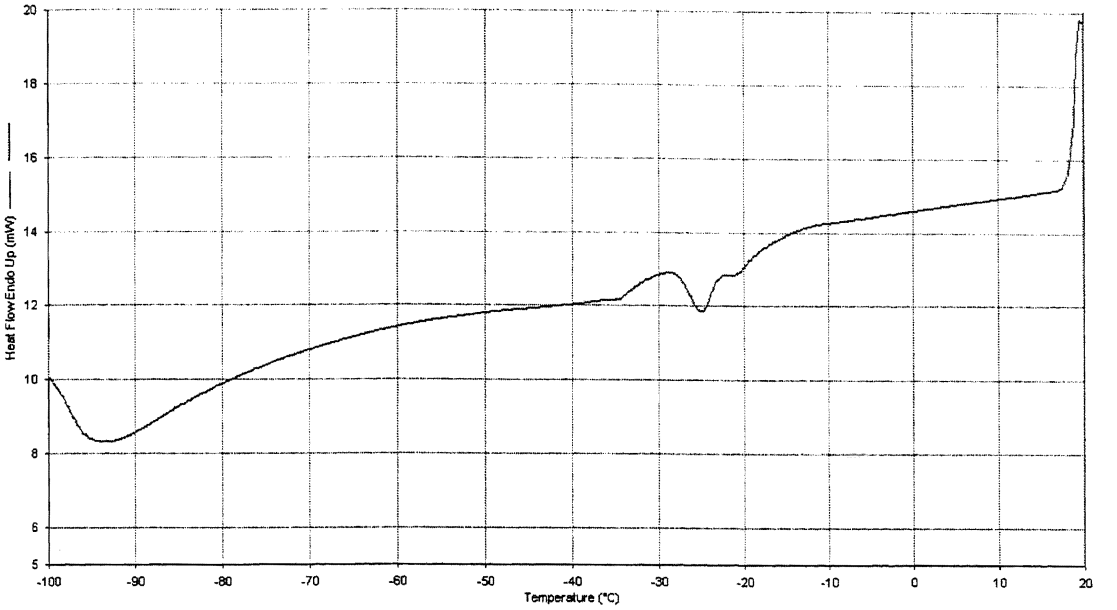


Fig. 3.9.2. The cooling cycle of *P. pinea* embryos of 5 % MC, showing peaks one likely to be lipids and the other glass transitions (tg).

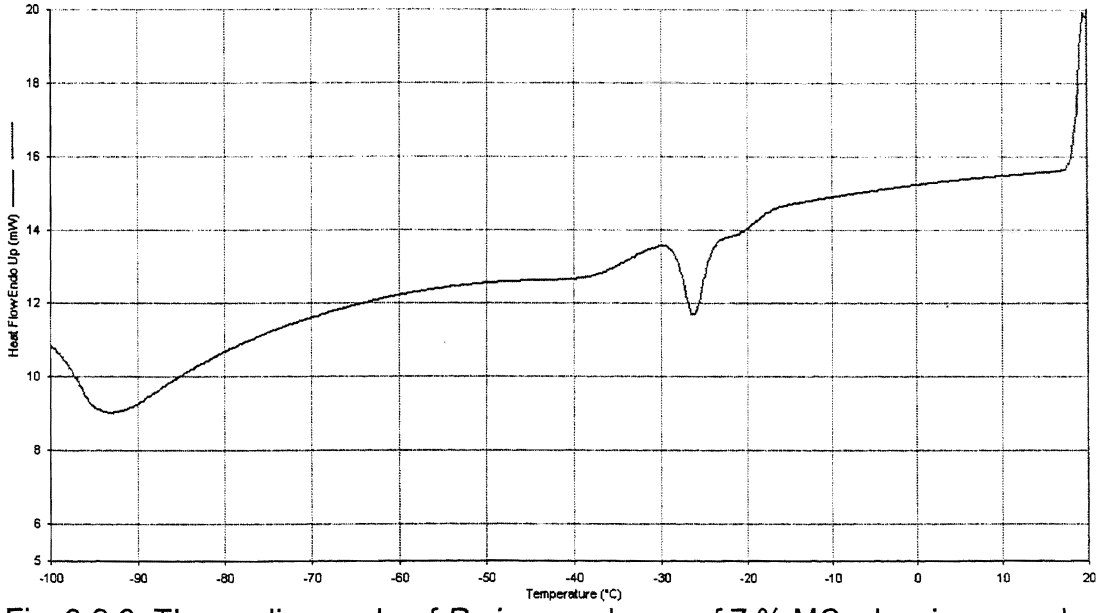


Fig. 3.9.3. The cooling cycle of *P.pinea* embryos of 7 % MC, showing a peak indicating glass transitions (tg).

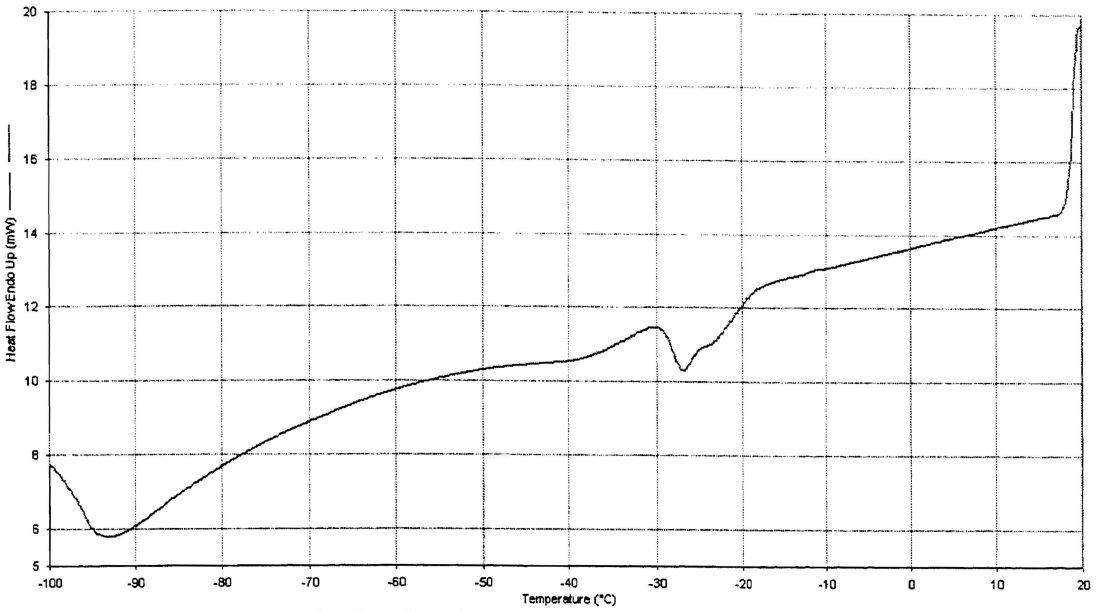


Fig. 3.9.4. Cooling cycle for *P. pinea* embryos at 9 % MC indicating two small peaks one representing lipids and the other glass transitions (tg) between temperatures of -20 and -30 °C.

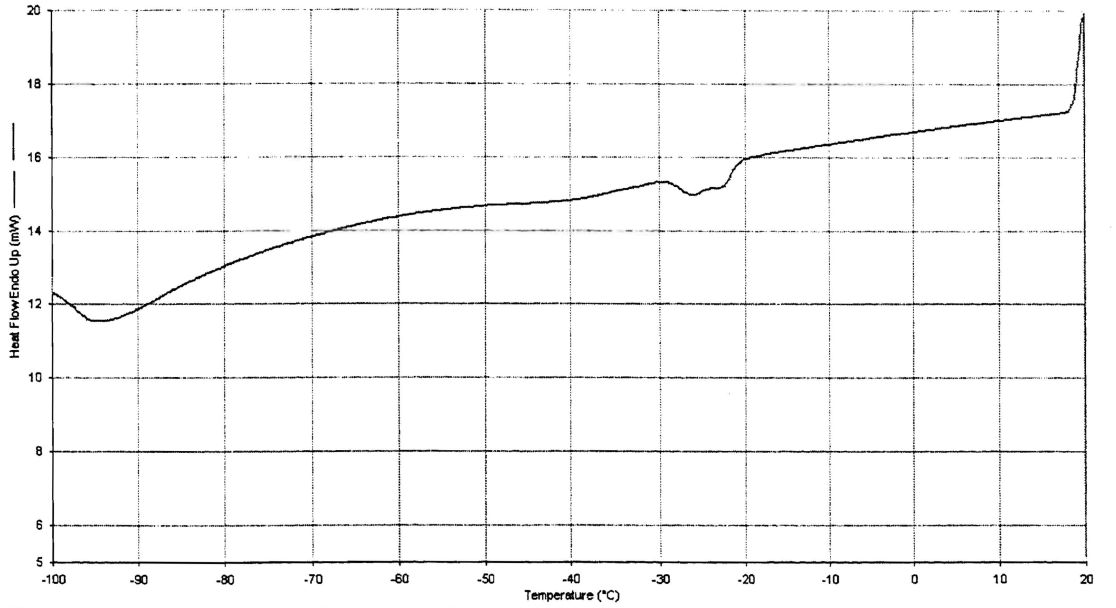


Fig. 3.9.5. Cooling cycle of *P. pinea* embryos at 11 % MC indicating two peaks, showing interaction between the two peaks one indicating glass transitions and the other lipids between temperatures of -20 and -30 °C.

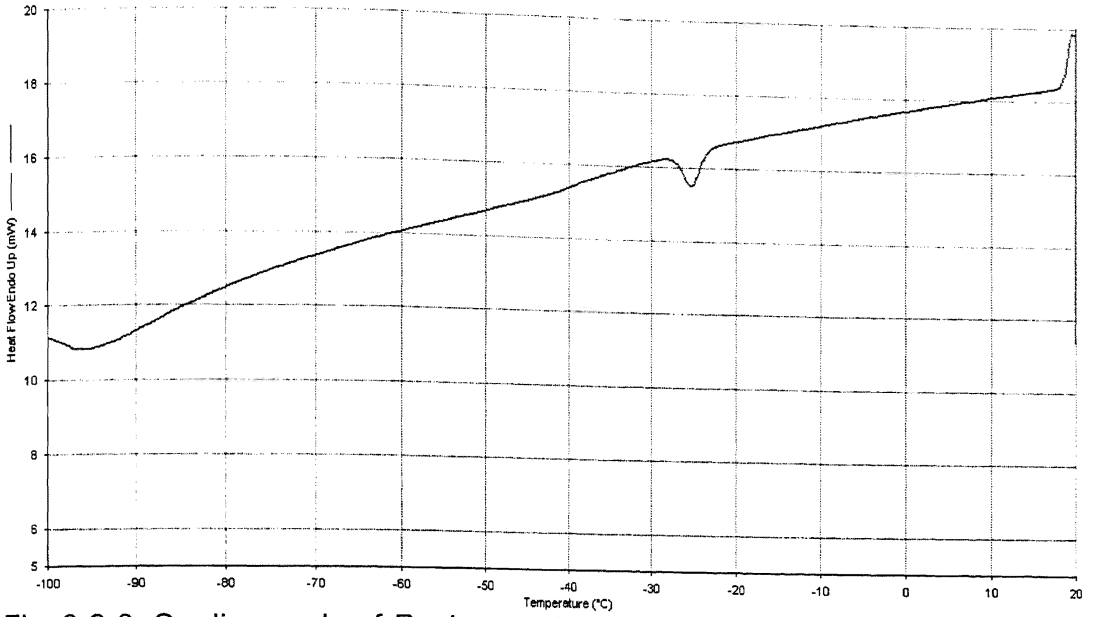


Fig. 3.9.6. Cooling cycle of *P. pinea* embryos indicating a small peak between -20 and -30 °C; indicating presence of lipids only.

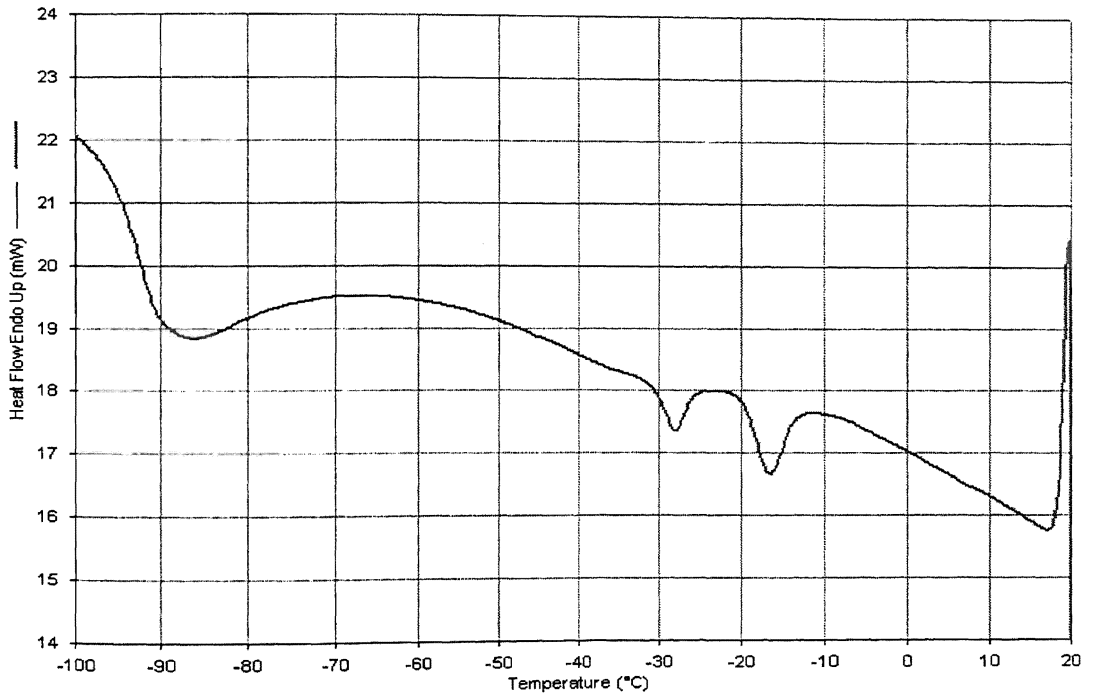


Fig. 3.9.7. Cooling cycle of *P.pinea* embryos of 15% MC showing two peaks which denotes interaction between water and lipids probably indicating ice formed at -18 and -28 °C.

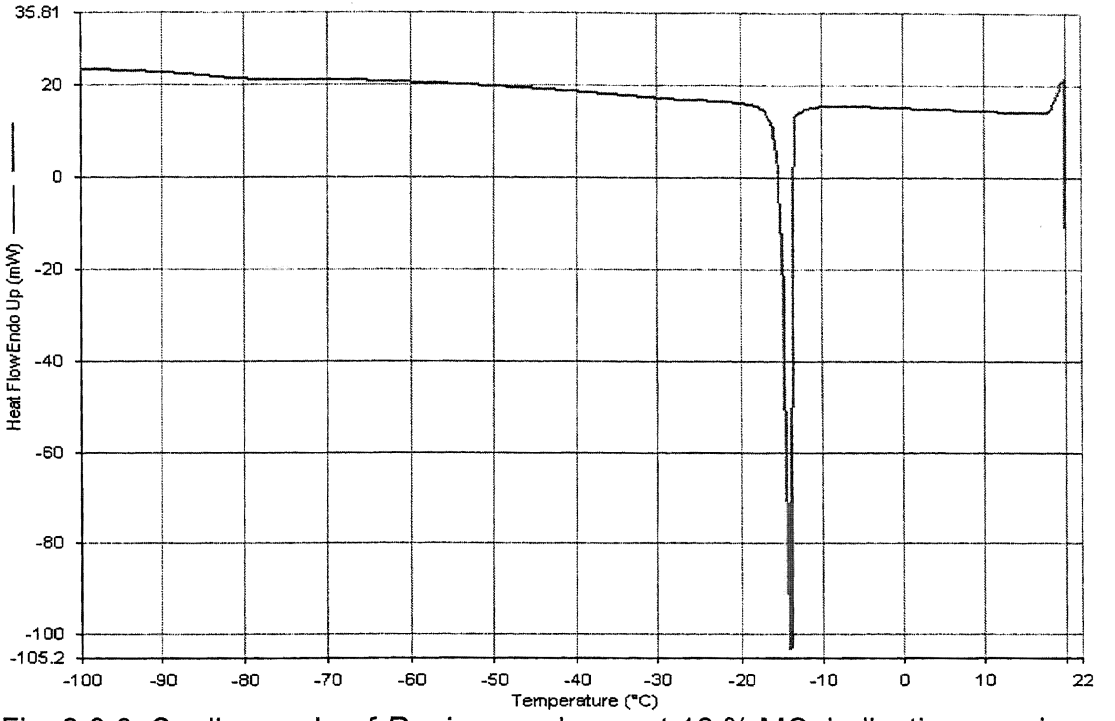


Fig. 3.9.8. Cooling cycle of *P. pinea* embryos at 18 % MC, indicating a major peak at -14 °C indicating ice formed at that temperature.

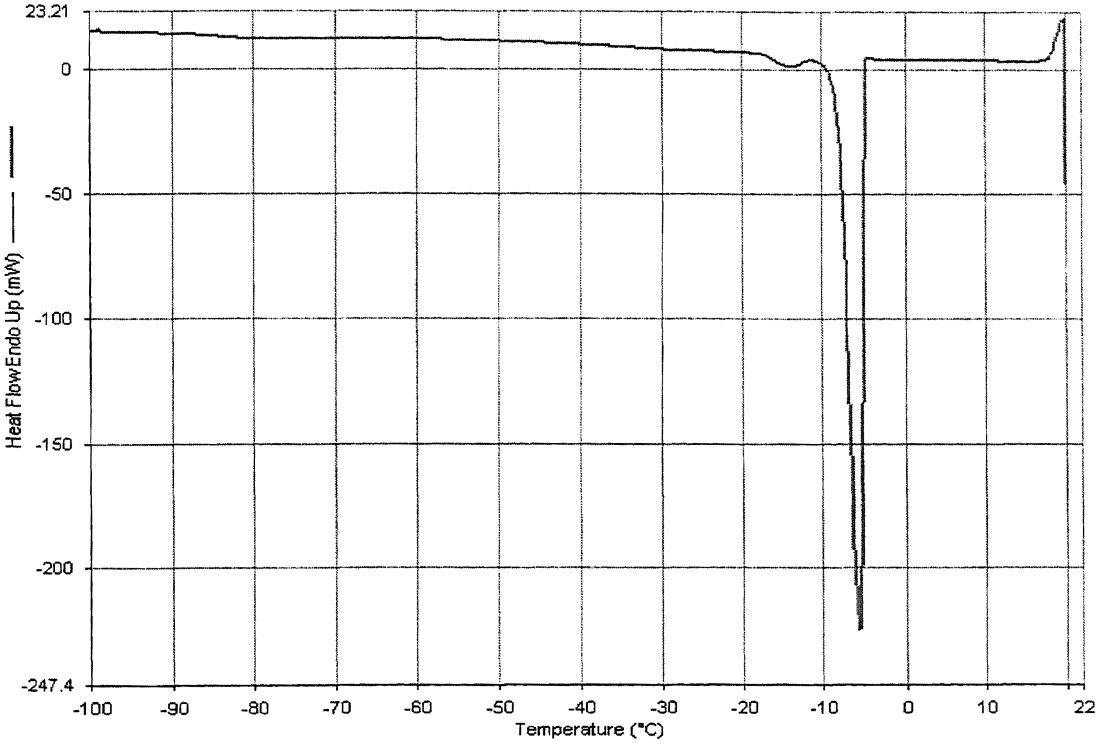


Fig. 3.9.9. Cooling cycle of *P. pinea* embryos showing peak formed at temperature of -8 °C indicating the formation of ice at that temperature.

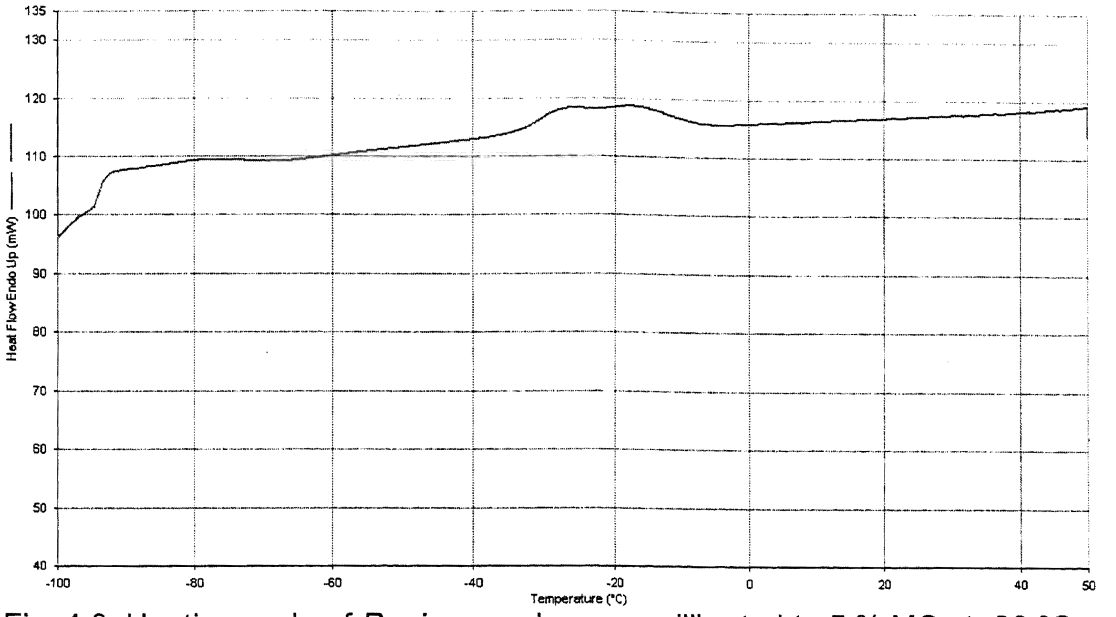


Fig. 4.0. Heating cycle of *P. pinea* embryos equilibrated to 5 % MC at -20 °C showing peak indicating presence of no ice crystals.

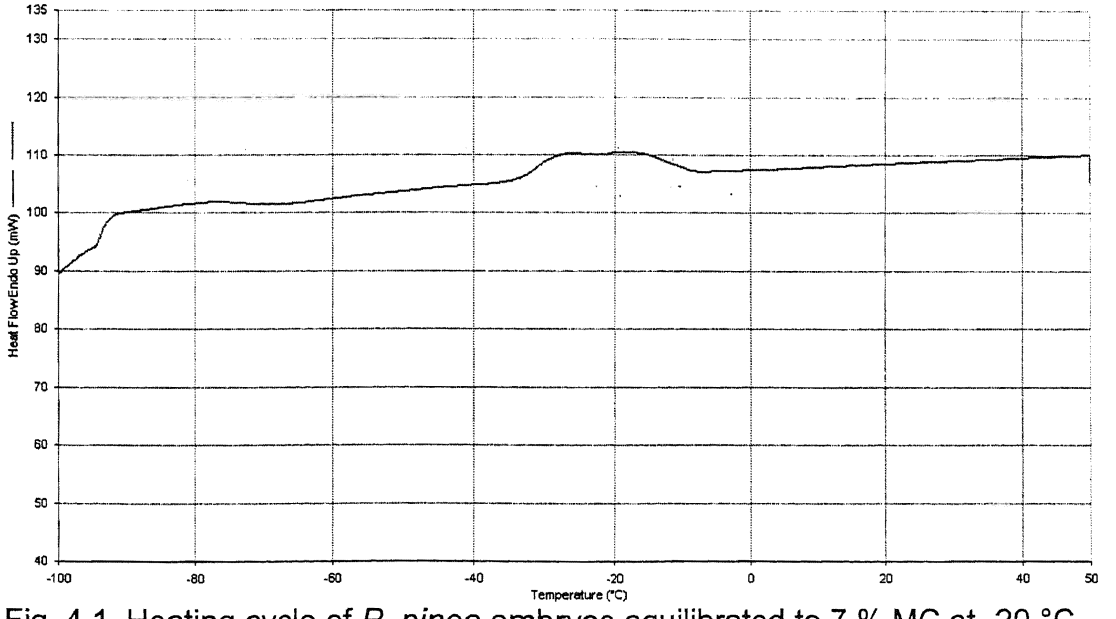


Fig. 4.1. Heating cycle of *P. pinea* embryos equilibrated to 7 % MC at -20 °C, showing peak indicating the presence of no ice crystals.

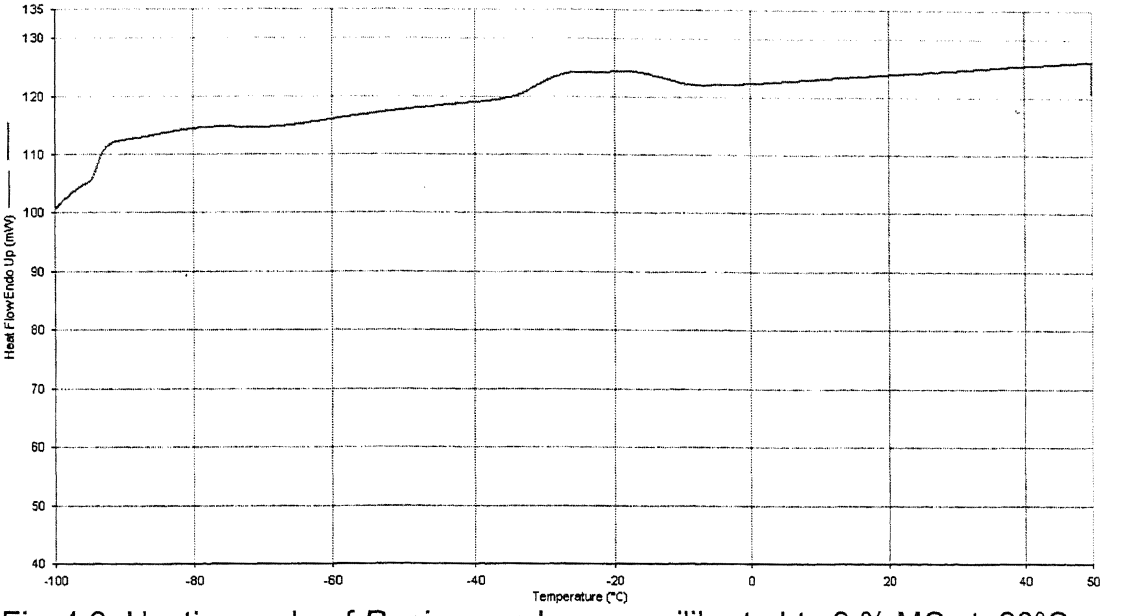


Fig. 4.2. Heating cycle of *P. pinea* embryos equilibrated to 9 % MC at -20°C showing peak indicating the presence of no ice crystals.

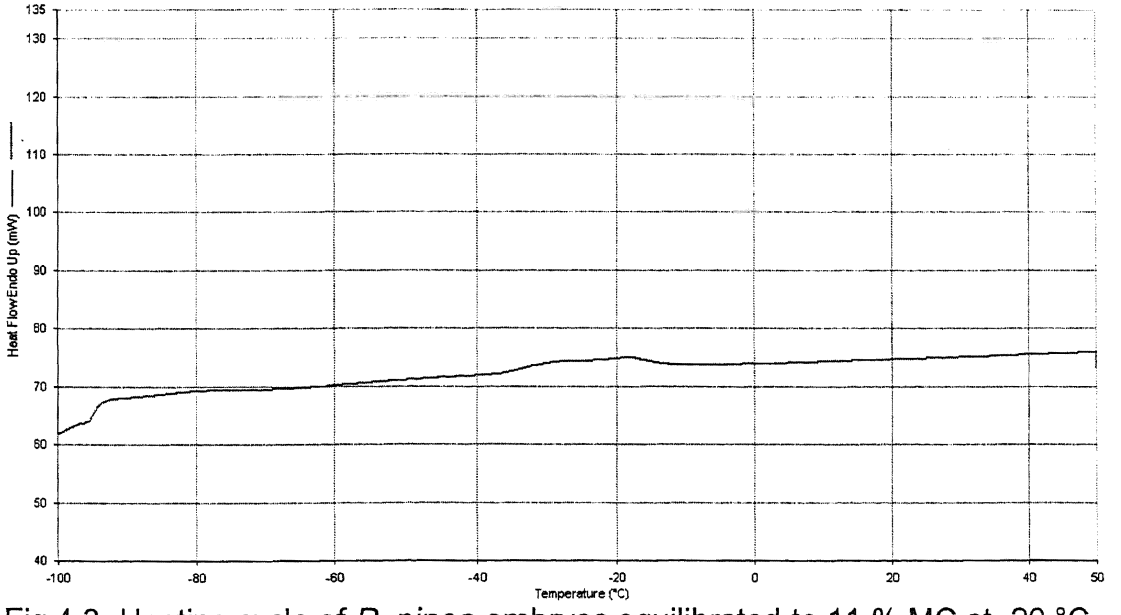


Fig.4.3. Heating cycle of *P. pinea* embryos equilibrated to 11 % MC at -20 °C showing peak indicating the presence of no ice crystals.

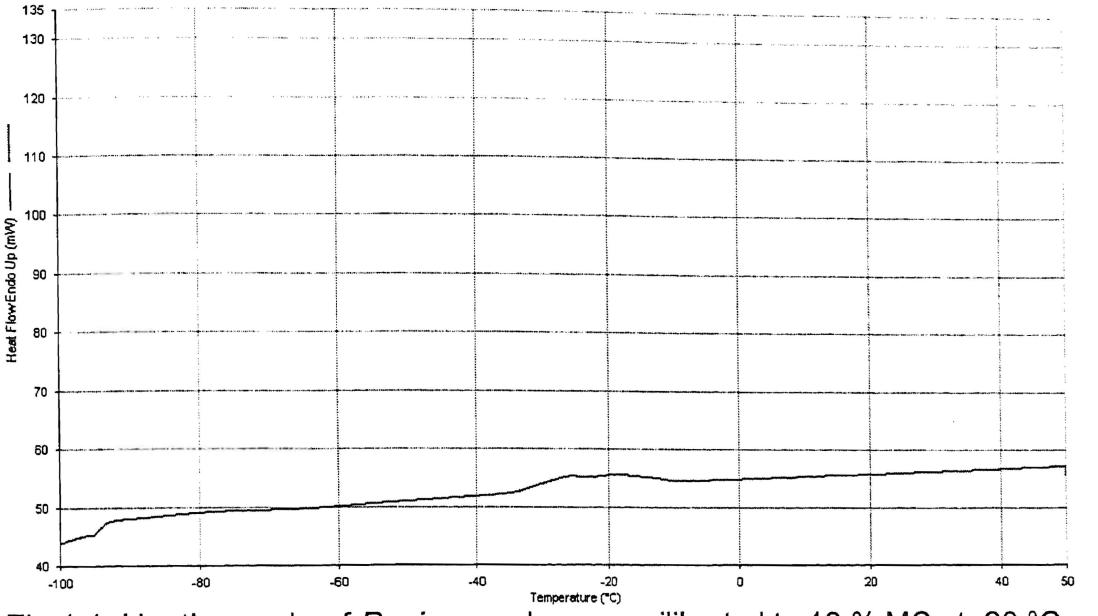


Fig.4.4. Heating cycle of *P. pinea* embryos equilibrated to 13 % MC at -20 °C showing peak indicating clearly the presence of no ice crystals but glass transitions.

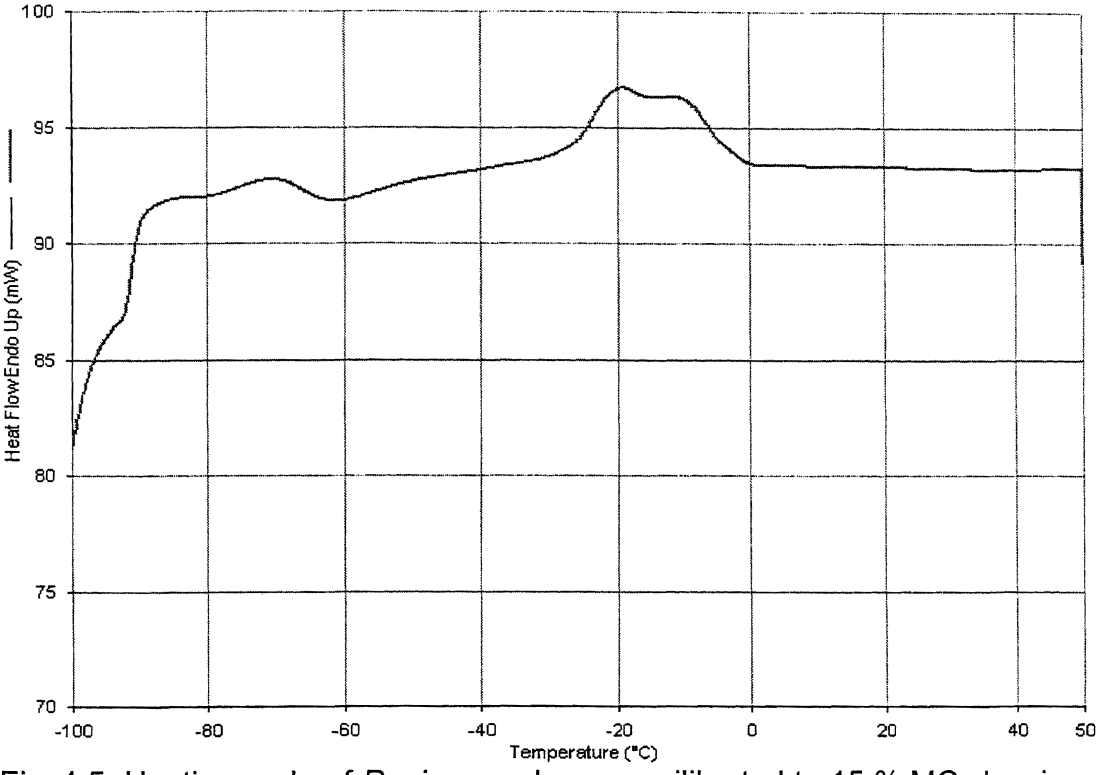


Fig. 4.5. Heating cycle of *P. pinea* embryos equilibrated to 15 % MC showing two peaks at approximately -20 °C stating the presence of lipids and water interacting, probably indicating presence of ice crystals.

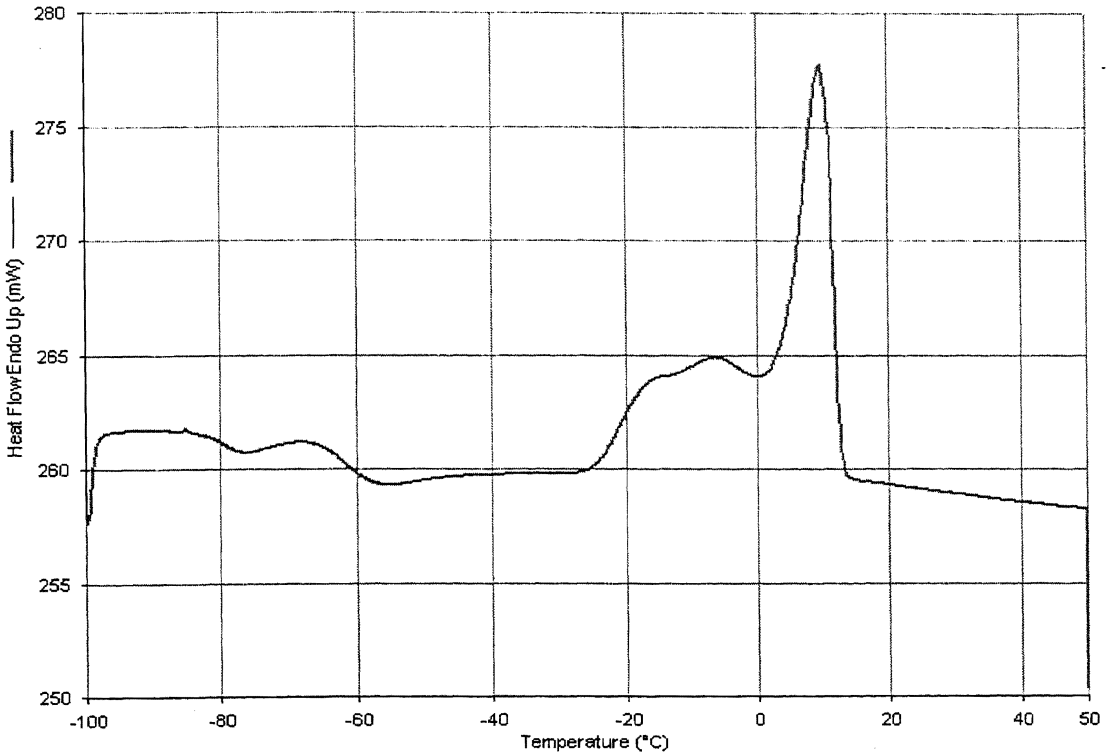


Fig.4.6. Heating cycle of *P. pinea* embryos equilibrated to 18 % MC at about 8 °C, showing sharp peak indicating the presence of ice crystals.

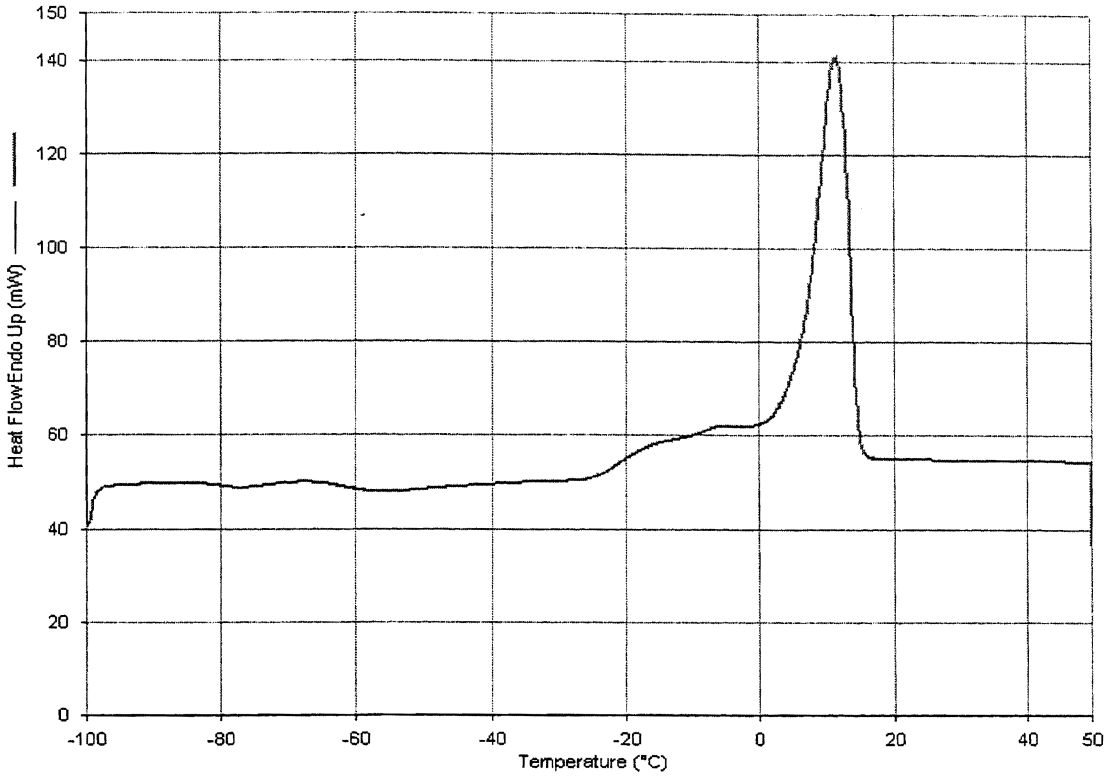


Fig.4.7. Heating cycle of *P. pinea* embryos equilibrated to 26 % MC, showing sharp peak formed on recrystallization of ice at temperature of 8 °C

Moisture content (%)	Replicate	Enthalpy Jg^{-1}
5	1	25.715
	2	19.111
7	1	24.849
	2	25.690
9	1	25.824
	2	19.860
11	1	21.456
	2	20.051
13	1	16.045
	2	36.921
15	1	21.778
	2	27.308
18	1	63.007
	2	77.277
26	1	105.092
	2	99.891

Table 3.5. DSC results for *Pinus pinea* embryos.

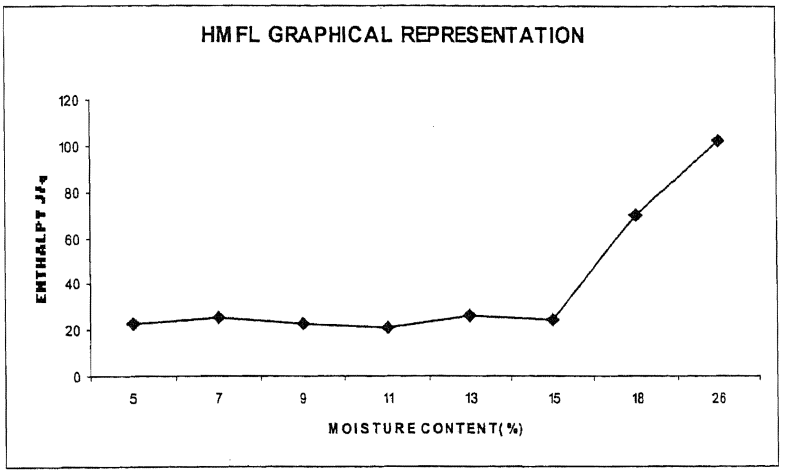


Fig. 3.9.1.1. High moisture freezing limit of *P. pinea* seeds ranging from 5 to 26 % MC.

CHAPTER FOUR

DISCUSSIONS AND CONCLUSIONS

4.0. Differences between the three species of pines

The four areas studied were:

- Germination of dried and treated seeds.
- Influence of moisture content on seeds and embryos of *P.pinea* seeds.
- Leakage in *P.pinea* and *P.caribaea* seeds.
- Chemical composition of pine seeds.

The results from the four areas studied showed that there were differences amongst the three pines.

4.1. Germination of dried and treated seeds

Dried seeds of *P. pinea, P. pseudostrobus* and *P. caribaea* were sown dry on agar for 28 days; and seeds of *P. pinea* seeds gave the highest percentage germination (93.75 %), followed by *P. caribaea* (66.25 %) and *P. pseudostrobus* seeds (48.75 %).

Cryopreservation studies showed that in *P. pinea* seeds 100% germination was achieved with T6 protocol which was significantly different from control, but not significantly different from T1, T4 and T5 (Table.3.1). In the case of *P. pseudostrobus* seeds before and after cryopreservation there was no significant difference between its control and all six treatments, although there was numerical trends in percentage germination indicating that T2 was the highest it was not significantly different from T4, T5 and T6 thus making these four treatment suitable for germination of this specie of pine. Interestingly,

while T4 is not significantly different from T1 and T3, T1 and T3 are significantly different from T2, T5 and T6.

In *P.caribaea* seeds, there was no significant difference between the control and treatments T1, T2, T3 and T4, of which T4 was numerically the highest .

Optimal survival following cryopreservation was achieved with different protocols for the 3 species. There were interesting differences in the impact of the six cryo-protocols used with the three species of pines including (i) T2 which was one of the lowest percentage germination in *P. pinea* seeds had one of the highest percentage germination in *P. pseudostrobus* seeds. (ii) In *P.caribaea* seeds T6 was one of the least suitable, but one of the most suitable protocol in the germination of *P.pinea* seeds.

Progressive profile germination curves show that treatments with the highest germination percentage seed-lots tends to germinate in a shorter period of emergence time as compared to seed-lots with lower percentage germination which tends to germinate with longer period of emergence time (Demir *et al.*, 2005). (compare Table 3.1 and Fig.3.4, 3.5 and 3.6).

In extended storage studies of *P.caribaea* seeds, seeds stored for 2 days to 24 days were not significantly different from one another as compared with the control with the exemption of seeds stored for 28 days which showed some stress in germination. In essence it is suggested that storage of this seed specie in liquid nitrogen(*P. caribaea*) for 28 days or more may be detrimental.

4.2. Influence of moisture content on seeds and embryos of *P. pinea*

Following the findings of *Pita et al.* (1998) where *P. pinea* seeds with 9.82 % moisture content stored in liquid nitrogen for 4 days resulted in a decline in seed germination; survival studies were carried out to determine whether moisture content was a factor responsible for the decrease in germination following storage in liquid nitrogen. Stanwood (1985) stated that the critical water content of orthodox seeds is between 10 - 30%, hence, the reason to determine if the moisture content compromises *P. pinea* seed survival.

Seeds of *P. pinea* were equilibrated to different moisture contents to derive an isotherm for *P. pinea* (Fig. 3.9) and 20 seeds in 4 replicates (totalling 80 seeds) were stored in liquid nitrogen for 4 days prior to sowing on agar for 28 days, and it was noticed that there was a decrease in survival in seeds with a percentage moisture content (fw) from 12 %. This suggested that *P. pinea* seeds with percentage moisture contents (fw) from 12 % MC would experience decrease in germination when stored or cryopreserved at sub zero temperatures.

Results obtained from this study indicate that at the moisture content (9.82 %) of *P. pinea* seeds used by Pita *et al.* (1998) which brought about the reduced survival of *P.pinea* seeds cannot be explained by moisture content alone.

4.3. Leakage in *P. pinea* and *P. caribaea* seeds

Increased solute leakage from seeds has been linked to decreased germination for many years. Fick and Hibbard (1925) reported that reduction in the resistance of leachate soak from timothy and red clover seeds was associated with decrease in germination. Leakage from single seeds was proposed as a method of predicting germination (Steere *et al.,* 1981; Midrad *et al.,* 2006). Thus any seed having a conductivity greater than a critical partition value was said to be ungerminable. These statements therefore shows the need to conduct leakage tests on seeds.

Seeds of *P. pinea* and *P. caribaea* was determined to ascertain whether leakage was a marker of cryo-stress in these two species. These pines were selected due to the decreased germination noticed with the T6 protocol in *P. caribaea* which was one among the lowest germination percentage among all the treatments. In contrast, with *P. pinea* T6 was one among the protocols, that gave highest percentage germination statistically. In *P. pinea* seeds the T2 protocol had the lowest percentage germination and showed the highest leakage (Fig 3.7) suggesting that leakage might be a stress marker in *P. pinea* seeds. However in *P. caribaea* seeds T5 and T6 had the lowest percentage germination but did not have the highest leakage (Fig 3.7.1) which was shown by T1; indicating that leakage is probably not a marker of cryo-stress in *P. caribaea* seeds.

4.4. Chemical composition of pine seeds

Another factor that might be responsible for the post cryopreservation decrease in survival of pines is their chemical composition. It is known that pine seeds are rich in oil (31 to 68 % by weight) (Wolff and Bayard, 1995) mainly fatty acids (Imbs and Pham, 1996) and in *P. pinea* the fatty acid content is close to 50 % (Carvalho, 1996).

The lipid rich nature of pine seeds increases the risk of cryo-damage because lipid thermal transitions can enable nucleation of small ice crystals forming into larger pernicious ones (Dussert *et al.*, 2001).

It is hypothesized that it is the interaction between lipid and water during freezing that is responsible for the formation of ice crystals large enough to cause lethal damage (Vertucci, 1989). Comparing lipid phase behaviour and seed survival in *Cuphea* species following exposure to -18 °C suggested that seeds were damaged because their lipids were in a crystalline state.

Differential scanning calorimetry tests confirmed that embryos of *P. pinea* equilibrated to moisture contents of 5, 7, 9, 11 and 13 %; water and glass transitions were observed through their cooling and heating thermograms (Figs 3.9.2 - 4.4) while at 15 % MC (fw) interaction of water and lipids became evident which could give rise to damaging ice crystals.

At 18 and 26 % MC (fw) cooling and heating thermograms (Figs 3.9.8, 3.9.9, 4.6 and 4.7) showed exothermic and endothermic sharp peaks at temperature of -20 °C indicating the presence of potentially lethal ice crystals.

The crystalline nature of lipids may cause damage in seeds (Walters *et al.,* 2002).

4.5. IMPROVEMENTS TO BE MADE ON THIS PROJECT

Due to time constraint and other factors two areas of study would have been worked on and if given the chance they would be

- Determination of optimum warming or thawing rates of these six treatments.

- The need to determine oil content for the three pine species and their thermal behaviour to enable better assessment of link between cold temperatures, oil transitions and germination survival in seeds.

4.6. CONCLUSIONS

The results of the investigations made on the succesful cryo-storage of the three pines (*P. pinea, P. pseudostrobus* and *P. caribaea)* show that:

- Moisture content of orthodox seeds, in pine seeds, especially *P.pinea* seeds, is not a major determinant of survival and that the moisture content of *P.pinea* seeds (Pita *et al.,* 1998) of 9.82%, though closer to critical moisture content of 10-30% (Stanwood, 1985), is not the cause in the decrease of survival of those seeds.
- Pre-cooling treatments (stepwise freezing treatments) proved to be alternative optimum protocols as compared to direct plung in liquid

nitrogen; as in T1, T4, T5 and T6 with *P.pinea,* T2, T4, T5 and T6 with *P.pseudostrobus* and T1, T2, T3 and T4 with *P.caribaea* seeds.

- Treatments with the highest percentage germination did not necessarily show the highest germination rates.
- Optimal survival of cryopreservation was achieved with different protocols for the three species of pine.
- Leakage detected in control and cryopreserved seeds of *P. caribaea* was not a stress marker indicating decrease in viability, while in *P. pinea* it does appear to be a stress marker.
- In the studies of differential scanning calorimetry (DSC) thermogams, storage of *P. pinea* embryos and possibly seeds at 5 - 13% MC was not detrimental to their survival, while storage between moisture contents (fw) of 15 and 26 % or more will be lethal.

4.7. Future work

Three areas should be considered to ascertain why some of the cryo-protocols explored did not yield high recovery in percentage germination in either *P. pseudostrobus* or *P. caribaea*:

- Seed oil content (Wolff and Bayard, 1995) or lipids interaction with water (Vertucci, 1989) of which lipid thermal transitions possibly enable nucleation of small ice crystals. DSC analysis of lipid content and water in a seed to determine relative concentrations that would lead to lethal damage.
- Genetic make up of the seed before cryopreservation or cryo-storage is explored; checking whether there are chromosomal abberations (Barnet and Vozzo, 1985) which can cause poor germination.

- In designing new protocols for storage in sub zero temperatures, the use of cryoprotectants should be explored before and during storage.

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